Functional activities of C-reactive protein on neutrophils

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ABSTRACT

C-reactive protein (CRP) is a member of the pentraxin family which opsonises *S. pneumoniae* through its binding to phosphorylcholine. In addition, CRP binds to Fcγ receptors: FcγRI with high affinity and, probably, FcγRIIA with lower affinity. Binding to FcγRIIA was suggested to depend on a polymorphism at residue 131 of the receptor, HH individuals being poorer binders compared to RR. To clarify the role of CRP binding to FcγRIIA and functional activities upon interaction, neutrophils from HH and RR individuals were incubated with CRP in the presence or absence of pneumococci type 3. No difference in activation as determined by IL-8 synthesis, respiratory burst and phagocytosis were seen between HH and RR donors.

Since CRP increases its serum concentration up to 1000 fold above the normal level, we also compared acute-phase concentrations of CRP with normal levels for neutrophil functions. Normal ($\leq$10 μg/ml) and acute-phase (10 – 100 μg/ml) levels increased IL-8 production in the presence or absence of pneumococci. A similar pattern was seen for extracellular reactive oxygen. There was a five-fold increase in DHR oxidation in neutrophils when CRP at 10 μg/ml was used, although this was reduced when CRP at 50 and 100 μg/ml was used in both HH and RR donors. Recombinant CRP also gave a similar pattern in which higher MFIs for DHR oxidation were obtained for CRP at 10 μg/ml. On average a two–fold increase in phagocytosis of pneumococci serotype 3 was obtained for both type of donors for CRP at 10 μg/ml. This effect was also reduced at acute-phase levels of CRP. The down-regulatory effects of CRP are thus selective for certain responses but do not require phagocytosis since CRP at 50 – 100 μg/ml could also inhibit PMA activation.

The ability of CRP to bind to Fcγ-receptors using surface plasmon resonance was evaluated, but there was no evidence of CRP binding to FcγRIIA, RIIB or RIII. Serum amyloid P component showed strong binding to FcγRIII and weak binding to FcγRIIA and RIIB. A glycosylated form of the Fc receptor may be required for binding.

RESUMEN

La proteína C reactiva (CRP) es miembro de la familia de las pentraxinas. CRP opsoniza, en forma dependiente del calcio, el *Streptococcus pneumoniae* a través de la unión con el residuo fosforilcolina el cual es expresado en la pared celular de la bacteria. Además la CRP se fija a los receptores Fcγ con afinidad diversa; así se fija al FcγRI con alta afinidad y probablemente al FcγRIIA con afinidad más baja. La unión de la CRP al FcγRIIA se ha sugerido que varia de acuerdo al polimorfismo expresado en el residuo 131 del segundo dominio extracelular del FcγRIIA, los individuos que son homocigotos para arginina (RR) en dicha posición se fijan mejor a la CRP comparados con los que son homocigotos para histidina (HH).

Para clarificar la unión de la CRP al FcγRIIA y las actividades funcionales que surgen de esta unión, neutrófilos de individuos tanto HH como RR fueron cultivados con diversas concentraciones de la CRP en presencia o ausencia del *Streptococcus pneumoniae* serotipo 3. No hubo diferencia en la activación del neutrófilo determinada por la síntesis de IL-8, la explosión respiratoria y la fagocitosis entre los dos tipos de individuos (HH y RR).

Debido a que la CRP incrementa su concentración sérica hasta 1000 veces por encima de su valor nomal, tambié comparamos los efectos que concentraciones normales y concentraciones agudas de la proteína tuvieran sobre la función del neutrófilo. Concentraciones normales de CRP (menores de 10 μg/ml) y concentraciones agudas (entre 10 y 100 μg/ml) incrementaron la producción de IL-8 en presencia o ausencia del pneumococo. Este efecto fue más pronunciado cuando el IFNγ estaba también presente. Un patrón de aumento similar se observó con la síntesis extracelular de compuestos derivados del oxígeno.

Hubo un incremento de 5 veces en la oxidación de la dihidrorodamina (DHR) cuando la CRP se usó a 10 μg/ml, sin embargo ese efecto se redujo cuando se usó CRP a 50 y 100 μg/ml tanto en los neutrófilos de donantes HH como RR. La CRP recombinante también produjo un patrón similar en el que mas alta oxidación de la DHR se obtuvo al usar CRP a 10 μg/ml. Se observó un incremento al doble en la

fagocitosis del pneumococo serotipo 3 en ambos tipos de neutrófilos al usar CRP a 10 μg/ml. Este efecto se redujo en forma similar cuando se usaron concentraciones altas de la CRP. Por tanto, los efectos reguladores negativos de la CRP son selectivos para ciertas respuestas y no requieren fagocitosis dado que CRP a 50 y 100 μg/ml también inhibieron la activación promovida por PMA.

Investigamos la capacidad de la CRP para fijar receptores Fcγ usando el Biacore, pero no pudimos demostrar unión ni al FcγRIIA ni RIIB ni al RIII. La proteína Amiloide A sérica (SAP) mostró alta afinidad por el FcγRIII, pero baja afinidad por los receptores FcγRIIA y FcγRIIB. La forma glicosilada del receptor Fc puede ser requerido para demostrar unión en este tipo de experimentos.

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List of abbreviations

AA	Arachidonic acid
AAT	$Alpha_1$-antitrypsin
ABC-1	ATP-binding cassette transporter 1
ACD	Citrate dextrose solution
ADCC	Antibody-dependent cell-mediated cytotoxicity
AGP	$Alpha_1$-acid glycoprotein
APPs	Acute phase proteins
β2gpI	$Beta_2$ glycoprotein I
βGR	Beta glucan receptor
BSA	Bovine serum albumin
CAD	Caspase-activated deoxiribonuclease
CbpA	Choline binding protein A
CD	Clusters of differentiation
C/EBP	CCAAT enhancer binding protein
CGD	Chronic granulomatous disease
$cPLA_2$	Constitutive phospholipase A_2
CR1	Complement receptor 1
CR2	Complement receptor 2
CR3	Complement receptor 3
CR4	Complement receptor 4
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
DAF	Decay accelerating factor
DAG	Diacylglycerol
DC	Dendritic cells
DED	Death effector domain
DHR	Dihydrorhodamine 123
DIC	Differential interference contrast
DISC	Death-inducing signalling complex
dNTP	Deoxyribonucleoside triphosphate
EB	Ethidium bromide
EDTA	Ethylenediamine-N,N,N',N'- tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
F-actin	Filamentous actin
FADD	Fas- associated death domain protein
FcγRI	Fc gamma receptor I
FcγRIIA	Fc gamma receptor IIA
FcγRIIA HH	Homozygous presence of histidine in the position 131 of the FcγRIIA
FcγRIIA RR	Homozygous presence of arginine in the position 131 of the FcγRIIA
FcγRIIB	Fc gamma receptor IIB
FcγRIIC	Fc gamma receptor IIC
FcγRIII	Fc gamma receptor III
FcγRs	Fc gamma receptors
FCS	Fetal calf serum

FITC	Fluorescein isothiocyanate
fMLP	N-Formyl-methionyl-leucyl-phenylalanine
fMLP-R	N-Formyl-methionyl-leucyl-phenylalanine receptor
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glysosyl phosphatidyl inositol
HBSS	Hank's Balanced Salt Solution
HGH	Human growth hormone
HLA	Human leukocyte antigens
HRP	Horseradish Peroxidase
ICAM	Intercellular adhesion molecule
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-1RA	Interleukin1 receptor antagonist
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IP_3	Inositol 1,4,5-trisphosphate
$iPLA_2$	Inducible phospholipase A_2
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun NH_2-terminal kinase
kDa	kiloDalton
LDL	Low density lipoprotein
LFA	Leukocyte function-associated antigen
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB4	Leukotriene B_4
LytA	N-acetylmuramoyl-L-alanine amidase
LytB	Endo-β- N-acetyl-glucosaminidase
MAPK	Mitogen-activated protein kinases
MARCO	Macrophage receptor with collagenous domain
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
MCP-1	Monocyte chemoattractant factor 1
MIP-1α	Macrophage inflammatory protein 1 alpha
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MLCK	Myosin light chain kinase
MPO	Myeloperoxidase
MR	Mannose receptor
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium

NF-κB	Nuclear factor kappa B
NHS	N-hydroxy-succinimide
OD	Optical density
PAF	Platelet activating factor
PAMP	Pathogen associated molecular patterns
PAP-1	Phosphatidic acid-phosphatase-1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	PBS containing 0.05%Tween 20
PCh	Phosphorylcholine
Pcp	Pneumococcal capsular polysaccharides
PCR	Polymerase chain reaction
pCRP	Purified C-reactive protein
PE	Phycoerythrin
PI 3-K	Phosphoinositol-3 kinase
pIgR	Polymeric immunoglobulin receptor
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PLD	Phospholipase D
PMA	Phorbol 12-myristate-13-acetate
PMN	Polymorphonuclear cells
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PspA	Pneumococcal surface protein A
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
RANTES	Regulated on activation normal T cell expressed and secreted
rCRP	Recombinant C-reactive protein
RT	Room temperature
SA	Streptavidin
SAA	Serum amyloid A
SAP	Serum amyloid P
SDS	Sodium dodecyl sulphate
SHIP	SH2-containing inositol 5' phosphatase
SIRP	Signal regulatory protein
SOD	Superoxide dismutase
SR	Scavenger receptor
SR-A	Scavenger receptor, class A
SR-B1	Class B scavenger receptor type I
SRBC	Sheep red blood cells
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TBSC	TBS containing 1 mM $CaCl_2$
TBST	TBS containing 0.05% Tween 20
TdT	Terminal deoxynucleotidyl transferase
TEMED	NNN'N'-tetra-methylethylendiamine
TGFβ	Tumour growth factor beta
Th1	T-cell helper response 1
Th2	T-cell helper response 2
TLR	Toll-like receptor

TMB	Tetramethylbenzidine-
TNFα	Tumour necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
TRITC	Tetramethyl-rhodamine isothiocyanate
TSP	Thrombospondin
TUNEL	Terminal deoxynucleotidyl transferase -mediated biotinylated deoxyuridine-triphosphate nick-end labeling
VAMP2	Vesicle-associated membrane protein-2
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organization

1. INTRODUCTION

Human C-reactive protein (CRP) is an acute phase protein the concentration of which increases once an inflammatory response has initiated, in severe cases by up to 1000 fold. CRP has a number of effects on the immune system. Among its functions, it activates the classical complement pathway upon binding to C1q, helps in the clearance of apoptotic cells and material derived from necrotic cells such as nuclear components and opsonises micro-organisms such as *Streptococcus pneumoniae* and *Leishmania donovani* promoting their phagocytosis. This can occur through complement receptors or directly through binding to Fcγ receptors (FcγRs).

Of the major phagocytic cells, neutrophils are cells that actively participate in the inflammatory response removing debris and micro-organisms either opsonised or non-opsonised. However, neutrophils are powerful producers of oxidative radicals derived from the respiratory burst that occurs in order to eliminate various micro-organisms, which once liberated can cause damage to the surrounding tissue. For this reason, a chronic inflammatory response may be associated with continuous production of oxidative radicals and other factors that may contribute to the pathogenesis of some autoimmune diseases.

CRP and neutrophils could have many different opportunities to interact during the inflammatory response; therefore it is possible that CRP exerts different effects on the function of these cells under different conditions. CRP effects on phagocytic cells have been explored by many groups around the world; however, it is still not clear how the interaction occurs and what the outcome of the neutrophil response might be.

We have chosen the interaction of CRP with Fcγ receptors in human neutrophils as a way to explore the responses that different concentrations of CRP can have and to clarify which of these Fcγ receptors is preferentially used in the context of the function of the cell. *Streptococcus pneumoniae* has been chosen as the working model; however its complexity may at the same time influence the final outcome obtained in the function of the neutrophils.

1.1 *Streptococcus pneumoniae*

S. pneumoniae (pneumococcus) is a diplococcus, gram-positive, aerophilic bacterium which despite antibiotic treatment and an improved vaccine is still one of the major pathogens all over the world.

Pneumococci cause disease more frequently in both extremes of life, with young children and the elderly being the most susceptible to it. The respiratory tract is the primary organ affected and is the one which this bacterium uses to gain access to the rest of the body, for this reason its main mechanism of transmission is airborne. In children, diseases commonly associated with the pneumococcus include otitis media, sinusitis, pneumonia and meningitis.

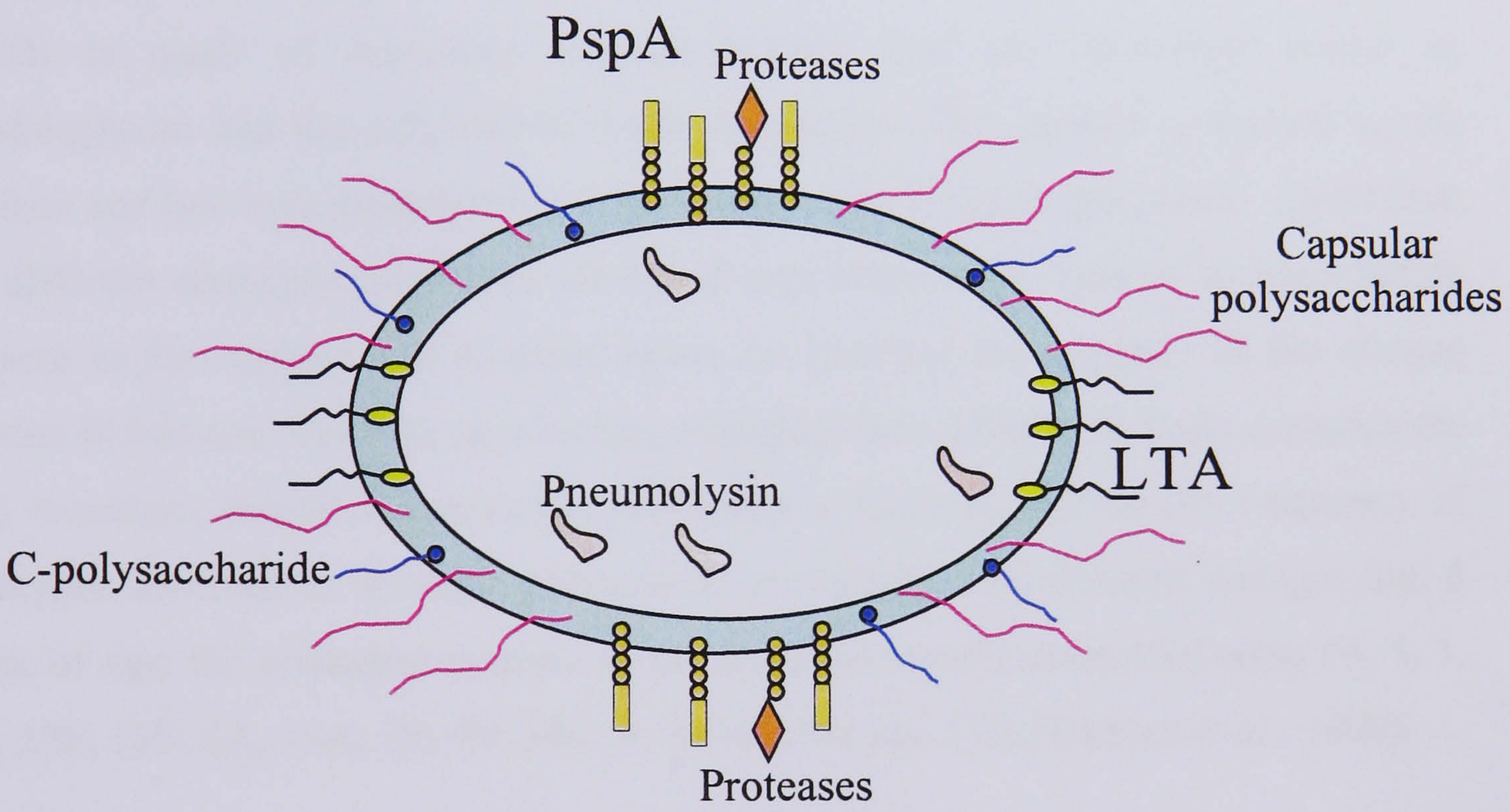


Figure 1.1 Structure of *Streptococcus pneumoniae*. Pneumococcus contains pneumolysin in the cytoplasm; LTA is formed from the cell membrane, whereas C-polysaccharide is the main structure found on the cell wall. On the surface some proteins such as pspA and proteases are present and finally a thick capsular polysaccharide surrounds the bacteria. LTA: lipoteichoic acid, pspA: pneumococcal surface protein A.

One child in three asymptomatically carries the pneumococcus in the nasopharynx at any given time. This bacterium is still a significant cause of acquired hearing loss resulting from both otitis media and meningitis. According to figures from the World Health Organisation (WHO), pneumococcus causes over 1 million deaths per year in

children younger than 5 years of age. In Europe and the USA pneumococcal pneumonia affects approximately 100 per 100000 adults each year. In these countries the mortality rate caused by pneumococcal pneumonia is 10% – 20% which may exceed 50% in the high-risk groups. (Anonymous, 1999)

This micro-organism has many different ways to deal with the complex defences of the immune system; thus, it has been able to survive through evolution. Some of the structures involved in pneumococcal pathogenesis include:

1.1.1 Capsule

Pneumococci are characterised by the presence of a thick polysaccharide capsule, which is made of repeating oligosaccharides that are covalently bound to peptidoglycan and the cell wall (C-) polysaccharide. The capsule is located on the surface and has been postulated to play a role in avoiding phagocytosis. More than 90 different serotypes have been identified according to the type of polysaccharide present in the capsule, but of these about 15 generate the majority of the disease burden in humans, the only species that pneumococcus affects. In Latin America the Pan American Health Organization developed a study to analyse the frequency of serotypes involved in invasive pneumococcal infections in children younger that 5 years of age; the serotypes in order of the most commonly associated were 14, 5, 1, 6B, 23F, 19F, 6A, 19A, 7F, 9V, 18C, 3, 4, 16F, 9N and 15B (Kertesz *et al.*, 1998).

The morphology of the colony also changes according to the thickness of the capsule: bacteria that colonise the nasopharynx and remain there for long periods of time appear not to have a capsule (transparent colonies) that facilitates binding to the epithelium through binding to platelet activating factor (PAF) receptor (Cundell *et al.*, 1995) or to polymeric immunoglobulin receptor (pIgR), whereas when the bacteria invades, capsule is present (opaque colonies), which among other factors allows dissemination of the bacteria. Capsule is required to prevent lysis or phagocytosis through complement, natural antibodies or pattern recognition receptors.

An interesting finding is that the pneumococcus can switch the type of polysaccharide expressed in the capsule if needed (capsular transformation), perhaps

as a way to avoid recognition by specific antibodies triggered by the polysaccharides present. It is known from studies *in vivo* and *in vitro* that penicillin resistance can be transferred from one bacterium to another by genetic exchange. DNA exchange can also be the cause for capsular transformation observed *in vitro* from clinical isolates and *in vivo* in both mice and man usually involving type 23 which is converted into types 3, 9N, 14 and 19F (Ottolenghi-Nightingale, 1972; Nesin *et al.*, 1998).

Although immune responses to the various components of the pneumococcus differ, traditionally capsular polysaccharide has been considered a T independent type 2 antigen (Pecanha *et al.*, 1991), this means that it is not presented in the context of MHC class I and also that the help from T cells required for the production of specific antibodies is minimal. Studies in mice, however, have challenged this concept by showing that CD40L expressed on $CD4^+$ T cells was essential for the production of the IgM and IgG antibodies specific for pneumococcal capsular polysaccharide (Jeurissen *et al.*, 2002). It appears that presentation on CD1 might be possible to induce recognition of polysaccharides by T cells. Dendritic cells pulsed with whole bacteria could promote certain isotypes of IgG for capsular polysaccharides when transferred into recipient mice (Colino *et al.*, 2002)

IgG antibodies to capsular polysaccharide are known to confer protection against invasive pneumococcal infections. These antibodies can recognise oligosaccharides expressed on the capsule, for that reason it is possible that these antibodies can cross-react with different pneumococcal serotypes expressing the same carbohydrate sequence. On the other hand, IgA antibodies to capsular polysaccharides might be effective at the mucosal level and thereby confer protective activities against otitis media caused by the pneumococcus.

In humans, it is clear that B cells require some signals for production of these anti capsular antibodies, and among them the interaction of CD40-CD40L appears to be of particular importance. Messenger RNA (mRNA) expression of CD40L determined by polymerase chain reaction (PCR) in peripheral blood mononuclear cells (PBMC), was increased after immunization with the 23-valent pneumococcal vaccine concomitantly with IL-4 (Leiva *et al.*, 2001), which helps in the synthesis of antibodies. Although CD40 ligation on B cells is normally associated with CD40L

expression by T cells, CD40L has also been reported on human dendritic cells (Pinchuk *et al.*, 1996); this mechanism could be useful to bypass T cell help (Wykes and MacPherson, 2000).

IgG1 and IgG2 antibodies confer protection against specific types of *S. pneumoniae* (Freijd *et al.*, 1984, Barrett and Ayoub, 1986) by opsonising the micro-organism and facilitating phagocytosis by neutrophils or macrophages (Gordon *et al.*, 2000). Individuals who lack specific antibodies against pneumococcal polysaccharides (specific antibody deficiency) (Sorensen *et al.*, 1998) or those who have IgG2 deficiency are susceptible to pneumococcal infections. With age, there is a tendency to have more IgG2 that IgG1 (Lottenbach *et al.*, 1999), the increased importance of IgG2 which binds better to FcγRIIA with histidine in position 131 implies that individuals with this polymorphism are less likely to get infection than those with arginine in the same position.

In the mucosa, other factors have been associated with resistance to the pneumococcus through binding to its capsular polysaccharide, e.g. IgA antibodies which in combination with complement components helps in opsonisation and phagocytosis of *S. pneumoniae* (Janoff *et al.*, 1999). Moreover, C3d fragments of complement associated with pneumococcal capsular polysaccharides have been found to bind CD21 (CR2 receptor) on B cells. This has the ability to increase the production of specific antibodies (Griffioen *et al.*, 1991). CD21 is expressed mainly by B cells and follicular dendritic cells on the marginal zone of the spleen (Peset Llopis *et al.*, 1996), cells which are lacking in patients who suffer from sickle cell disease or who have had a splenectomy, conditions that predispose to pneumococcal infections.

1.1.2 Cell wall and membrane

The cell wall consists of a peptidoglycan and teichoic acid. The peptidoglycan consists of long chains of alternating N-acetyl-D-glucosamine and N-acetylmuramic acid from which extend chains of four to six amino acids called stem peptides. C-polysaccharide is composed of two parts: 1) teichoic acid, which is able to protrude

into the capsule (Musher, 2000), is a phosphorylcholine (PCh) containing polymer of low molecular weight carbohydrates such as N-acetylglucosamine joined together through diester linkages to ribitol phosphate residues and; therefore, classified as ribitol teichoic acid and 2) fragments of peptidoglycan (Sorensen, 1995).

Presence of PCh on the pneumococcal surface confers ability to bind to CRP, myeloma proteins and PAF receptors (Bruyn *et al.*, 1992). PCh binding to PAF receptors is important since when PCh binding to PAF receptors was blocked, markers of inflammation such as leukocyte migration were decreased in a rabbit model of pneumococcal meningitis and pneumonia (Cabellos *et al.*, 1992). This finding is highlighted by the activity of pneumococcal phosphorylcholine esterase that removes up to 30% of the PCh present. When this enzyme is inactivated, an increase in the virulence of mutated pneumococci in a mouse model is observed, apparently due to more pneumococcal adherence to PAF receptors (Vollmer and Tomasz, 2001) or possibly due to an increased attachment of other virulence factors to the bacteria. However the clinical implication of this enzyme in clinical isolates remains to be seen. Adding to this, increased expression of PCh on *H. influenzae* was shown to lead to reduced virulence in rats (Humphries and High, 2002).

An immunogenic component of the membrane of the pneumococcus is lipoteichoic acid which together with teichoic acid is able to promote an inflammatory response by interaction with toll-like receptor 2 (TLR2) and CD14 (Yoshimura *et al.*, 1999). Proinflammatory cytokines induced by these molecules increase the migration of leukocytes to the affected tissue and are partially involved with symptoms associated with pneumococcal infections. IgG antibodies to cell wall polysaccharide were shown not to protect against the progression of acute pneumococcal infection from colonisation to acute purulent bronchitis, which is due to encapsulated bacteria (Musher *et al.*, 1990). Although this study did not analyse phagocytosis of opsonised pneumococcus with these antibodies, the implication is that antibodies directed to non-encapsulated bacteria do not protect against encapsulated pneumococcus.

1.1.3 Pneumolysin

Pneumolysin is a 53 kDa polypeptide member of the family of thiol-activated cytolysins ubiquitously present in all clinical pneumococcal isolates. This haemolytic toxin is able to lyse all cells that have cholesterol in their membranes and therefore it is one of the main virulence factors involved in pathogenesis (Mitchell, 1999). Pneumolysin is located in the cytoplasm and can be liberated after lysis of the micro-organism. Its gene was characterised in 1986 and mutants lacking the gene have been constructed, shown to have reduced virulence and used to analyse the role of this protein in the ability of the pneumococcus to cause infection (Berry *et al.*, 1989).

Pneumolysin activates the classical complement pathway in the absence of specific antitoxin antibodies, apparently upon non-specific binding to the Fc portion of antibodies. Complement activation was determined by detecting cleavage of C3 by radioimmunoassay. Mutations of pneumolysin in amino acid residues 384 and 385 cause a decrease in the ability of the toxin to bind antibody and hence activation of the classical pathway (Mitchell *et al.*, 1991).

Previously pneumolysin was reported to decrease the respiratory burst of neutrophils (Paton and Ferrante, 1983), however, recent studies with recombinant pneumolysin have shown increased phospholipase A2 activity, associated with increased expression of CR3 and enhanced superoxide production. These effects were explained by an increased calcium influx due to a pore forming mechanism (Cockeran *et al.*, 2001). Recently, pneumolysin has been shown to induce production of TNFα and IL-6 by macrophages through TLR4. Mice with a mutation on TLR4 were more susceptible to pneumococcal infection than wild-type mice (Malley *et al.*, 2003). These data suggest that pneumolysin also contributes to the innate immune response against pneumococci.

Pneumolysin has been evaluated for its ability to act as a pneumococcal vaccine in mice. Alexander *et al.*, (1994) used mutated pneumolysin to induce production of IgG1, different mice strains were then challenged against nine different pneumococcal serotypes showing some protection to most of the serotypes used,

although responses were variable among the mice used (Alexander *et al.*, 1994). Pneumolysin and choline binding protein A (CbpA) or the combinations of both have also been tested in mice providing better survival when the combination is used (Ogunniyi *et al.*, 2001). In humans, a conjugated vaccine that involves both a pneumococcal capsular polysaccharide and pneumolysin might be useful since both components are from *S. pneumoniae* whereas currently conjugated vaccines use the pneumococcal polysaccharide conjugated to a protein from a different micro-organism.

1.1.4 Surface proteins and enzymes

Proteins expressed in the surface of the pneumococcus may be either anchored via the attachment motif LPXTG and protein attached to peptidoglycan (transpeptidase activity) (Lee *et al.*, 2002). Alternatively, they may be non-covalently associated with choline; these proteins are called choline binding proteins and include CbpA, PspA and N-acetylmuramoyl-L-alanine amidase (LytA) (see below). Some enzymes like neuraminidase enzymes and hyaluronidase also contain the LPXTG motif. (Mitchell, 2000).

CbpA is important for adherence of the bacteria to the nasopharyngeal epithelium as has been shown for transparent colonies, which have an increased expression of this protein (Kim and Weiser, 1998). Interestingly, CbpA binds to the secretory component of IgA (Hammerschmidt *et al.*, 1997), probably interfering with IgA function, but also it might be associated with binding to pIgR.

PspA is a lactoferrin-binding protein ((Hammerschmidt *et al.*, 1999), (Hakansson *et al.*, 2001)) that allows the bacteria to have access to iron that is captured by lactoferrin found in human milk or produced by neutrophils. In addition, this protein has been shown to inhibit complement activation by *S. pneumoniae* (Tu *et al.*, 1999).

Enzymes that participate in the pathogenesis of invasive pneumococcal infections allowing disruption of the connective tissue include neuraminidase enzymes (NanA and NanB) and hyaluronidase that are able to induce local inflammation. Models that use pneumococcal strains with mutated forms of these enzymes have shown less virulence when compared to the wild type strains (Mitchell, 2000).

Autolysins are enzymes that degrade the cell wall of micro-organisms. Based on the chemical bond that these enzymes break down in the peptidoglycan substrate, they are classified as glycosidases (muramidases or lysozymes, glucosaminidases and transglycosylases) amidases or endopeptidases (Garcia *et al.*, 1999b). One of the first characterised autolysins of the pneumococcus is LytA which is a choline-binding protein (Garcia *et al.*, 1999a).

Presence of choline residues in the cell wall teichoic acid is essential for the sensitivity to LytA, LytB (endo-β- N-acetyl-glucosaminidase) and LytC; by the action of these enzymes, the cell wall is fragmented and each fraction becomes highly proinflammatory, for example inducing production of IL-1, IL-6 and TNFα by human monocytes (Riesenfeld-Orn *et al.*, 1989; Heumann *et al.*, 1994).

Some of these proteins have been suggested as potential candidates for vaccination, however the practical effectiveness for this or other strategies remains to be seen. In the meantime, the pneumococcus continues to be an important human pathogen.

1.2 Neutrophils

Neutrophils are granulocytes produced by the bone marrow in humans. The bone marrow weighs about 2500g as compared to liver which weighs 1500g; up to 60% of the cell production of the bone marrow is dedicated to them with the result that they are the most numerous blood cell. Neutrophils are characterised by nuclei segmented into 3 to 5 interconnected lobes, hence they are known as polymorphonuclear (PMNs) cells. Also because of the presence of multiple granules in their cytoplasm they are also considered a type of granulocyte (Bainton, 1999). Once in the circulation, the half-life of neutrophils is approximately 10 hours, but once they reach the tissue they can live up to 2 days. To reach its maximum maturation neutrophils start from a myeloblast stage, which contains no granules, are transformed into a promyelocyte, then myelocyte, metamyelocyte, bands and finally the mature form.

During acute inflammation the release from bone marrow is increased to generate the leukocytosis seen. This is under the control of cytokines such as G- and GM-CSF which, in conjunction with IL-1β and TNFα, influence the production of these cells in the bone marrow and are associated with the accumulation of neutrophils in the lungs and other tissues observed with different stimuli such as LPS and IL-17 (Laan *et al.*, 2003)

During these changes the neutrophil acquires the characteristic primary or azurophilic granules named because of their affinity to azure dye. Primary granules appear for the first time in the promyelocyte stage which are characterised by the presence of myeloperoxidase. Thereafter, secondary or specific granules appear which contain lactoferrin and collagenase among other substances important in the function of these cells. Tertiary or gelatinase granules have a high concentration of this enzyme and secretory vesicles that contain plasma proteins endocytosed by neutrophils (Table 1.1) (Burg and Pillinger, 2001).

Elie Metchnikoff first described neutrophils at the end of the nineteenth century from their ability to phagocytose. It is now clear that neutrophils are not only phagocytic cells, but they have also the capacity to influence the inflammatory response and are important in connecting innate immunity with the adaptive immune response through the production of MCP-1 and IFNγ. MCP-1 is important in delayed-type hypersensitivity reaction and the production of IFNγ a key cytokine in the Th1 response (Yamashiro *et al.*, 2001). Neutrophils are the first leukocytes to go to an inflammatory site and they are the hallmark of acute inflammation.

Neutrophils synthesise a range of enzymes and substances that are effective in destroying ingested micro-organisms. However some secreted molecules have also an immunomodulatory role once released to the external environment. Some of these proteins are the neutrophil-derived serine proteases stored in the primary granules: elastase, cathepsin G and proteinase 3. These proteins are active at neutral pH and besides their ability to participate in the degradation of the extracellular matrix may also catalyse the activation of cytokines such as IL-1β and TNFα (Bank and

Ansorge, 2001). This is one example of other potential functions of neutrophils on the immune system besides their phagocytic capacity.

Some of the functional activities of neutrophils are as follows:

Chemotaxis: Neutrophil binds to a chemoattractant, which provide a chemical gradient that allows the cell to follow it. Many different substances have the ability to attract them including C5a (derived from complement activation), PAF, fMLP (only produced by bacteria), LTB4, and a number of chemokines of which IL-8 appears to be the more important (Walz *et al.*, 1987). Neutrophils express receptors for each of these chemokines to allow binding to them.

Migration: Chemotactic substances and different cytokines are able to induce the expression of adhesion molecules (integrins, selectins and immunoglobulin superfamily members) on both endothelial cells and neutrophils which facilitate the interaction between these cells. The expression of E-selectin on endothelial cells and L-selectin on neutrophils is important for rolling of the latter cells. This event has been called margination of neutrophils from free flow to the endothelium (Picker *et al.*, 1991).

If the stimuli (such as TNFα production) persists, the expression of adhesion molecules differs from the one described above. Neutrophils are induced to express β2-integrins, LFA-1 (CD11a/CD18) mac-1 (CD11b/CD18) and p150/95(CD11c/CD18) which are ligands for ICAM-1 and –2 expressed on activated endothelial cells; this allows a firm adhesion of neutrophils to the endothelium. Then, through a process still not well understood, the neutrophil extends pseudopods to migrate to the site where the chemotactic stimulus is being produced. For this late event, there is a role for some β1-integrins, such as α9β1, inhibition of which is able to block the migration of neutrophils to the tissue (Butcher, 1991).

Endocytosis and degranulation: The recognition of micro-organisms by neutrophils can be either direct through specific receptors or indirect, binding first to opsonins (Christiansen and Skubitz, 1988)

Table 1.1 Proteins found in human neutrophil granules and secretory vesicles. Modified from Bainton (1999).

	Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Membrane	CD63, CD68 V-type H+-ATPase	CD11b, CD15 CD66, CD67, TNF-R , fMLP-R Cytochrome b_{558} Fibronectin-R Laminin-R G-protein subunit Thrombospondin-R Vitronectin-R Rap1, Rap2 Urokinase-type Plasminogen activator -R	CD11b Cytochrome b_{558} Dyacylglycerol-deacylating enzyme fMLP-R Urokinase-type Plasminogen activator -R VAMP-2 V-type H+-ATPase	Alkaline phosphatase CR1, CD11b Cytochrome b_{558} CD14 CD16 fMLP-R V-type H+-ATPase
Matrix	Acid β -glycerophosphate Acid mucopolysaccharide α1- antitrypsin α- mannosidase β-glycerophosphate Heparin- binding protein Bactericidal permeability increasing protein β-glucuronidase Cathepsins Defensins Elastase, Sialidase Lysozyme Myeloperoxidase N-acetyl-β-glucosaminidase Proteinase-3	β2-microgloblin Gelatinase Lysozyme Collagenase Gelatinase Histaminase Heparanase Lactoferrin, Lysozyme Sialidase Vitamin B12-binding protein	Acetyltransferase β2-microglobulin Gelatinase Lysozyme	CD10 CD13 CD45 C1q-receptor DAF Albumin Tetranectin

Killing: Killing occurs as a result of a combined effect of superoxide anion production, the activity of proteases and possibly removal of factors needed for microbial growth or survival (Reeves *et al.*, 2002).

1.2.1 Cytokine production by neutrophils

Although many cytokines have been reported to be produced by neutrophils, these data should be considered with caution due to the contribution of contaminating eosinophils or basophils which are particularly difficult to separate from neutrophils. More recent papers based on FACS analysis of intracellular production have provided more reliable data.

Requirements for the production of these or other cytokines may vary from study to study, but it is clear that in most conditions production of these cytokines are higher after priming the neutrophils with a stimulant. That is, ability to produce a particular cytokine like TNFα may require pre-incubation with GM-CSF or IFNγ to respond to LPS or fMLP to a greater degree.

In some reports it is not clear which type of priming has been used, but even the type of blood anti-coagulation used may have an effect, for example the use of heparin might prime neutrophils since they express a receptor for it (table 1.1). Use of foetal calf serum (FCS) or other supplements during culture procedures may provide the elements required for priming.

Neutrophils are reported to synthesise proinflammatory cytokines such as IL-1β, TNFα and IL-6, are able to stimulate their own chemotaxis and activation through the production of IL-8 and may also have the capacity to influence the adaptive immune response by the synthesis of IFNγ which then may influence T cells to become Th1 (Yeaman *et al.*, 1998).

IL-8 is an autocrine cytokine produced by neutrophils in higher concentration compared to other proinflammatory cytokines. mRNA for IL-8 was found in non-

stimulated neutrophils whereas mRNA for IL-1β, IL-6 and TNFα only appeared after stimulation (Altstaedt *et al.*, 1996). IL-8 is a CXC chemokine initially shown to be synthesized by mononuclear cells (Matsushima *et al.*, 1988; Peveri *et al.*, 1988), later neutrophils were also shown to produce this chemokine (Bazzoni *et al.*, 1991). IL-8 promoted migration of neutrophils 4 – 6 hours after instillation into the skin of rabbits (Colditz *et al.*, 1990). However, IL-8 not only attracts neutrophils to the site of inflammation but also at higher concentrations induces their adherence, degranulation, respiratory burst and lipid mediator synthesis (Baggiolini *et al.*, 1994).

At high cell density IL-8 synthesis is regulated, after increasing neutrophils up to 6 x 10^7 cells/ml production of IL-8 was reduced concomitantly with the secretion of IL-1RA and soluble TNF receptor (sTNFR) (Hattar *et al.*, 2001). Another cytokine that can be immunomodulatory is TGFβ, which also has been shown to be synthesised by human neutrophils (Chu *et al.*, 2000). TGFβ1 does not activate neutrophils as shown by superoxide generation but it induces chemotaxis provoking polymerisation and redistribution of F-actin. This effect is neither dependent on influx of calcium nor GTPase activity (Reibman *et al.*, 1991).

Neutrophils are also reported to induce migration of mononuclear cells as has been shown by the secretion of MIP-1α. Different micro-organisms are able to induce the production of IL-8 or MIP-1α to different degrees: *Pseudomonas aeruginosa* and *Staphylococcus aureus* induced both IL-8 and MIP-1α; *Streptococcus pneumoniae*, *Staphylococcus epidermidis* and *Candida albicans* induced both but to a lesser extent; *Saccharomyces cerevisiae* and zymosan induced IL-8 but not MIP-1α. (Hachicha *et al.*, 1998).

1.2.2 Phagocytosis

Phagocytosis is a phylogenetically ancient response to particles adopted by phagocytes such as neutrophils and macrophages. It requires the recognition of small particles exceeding 0.5μm (the size of *Mycoplasma*, the smallest microbe known to stimulate phagocytosis), the formation of pseudopods which are extensions of the cytoplasm rich in filaments that are part of the cytoskeleton (e.g. Filamentous actin

(F-actin), vinculin and talin) and temperature, the lowest temperature that allows phagocytosis is between 13 and 18°C (Greenberg, 1999a).

In vivo before the real contact between the particle and the phagocyte certain steps have to be accomplished which briefly are chemotaxis and opsonisation of the particle. The recognition of micro-organisms by neutrophils can be either direct through specific receptors, for instance through scavenger receptors or indirect, with pathogen binding first to opsonins (Christiansen and Skubitz, 1988). Opsonins which include immunoglobulins, some complement fragments such as C3b and C4b and various acute phase proteins such as MBL and CRP, are very important in protection against encapsulated bacteria such as pneumococcus and any defect in these proteins may result in an increased frequency of bacterial infections (see sections 1.3.1 and 1.3.2).

Neutrophils contain specific receptors for opsonins such as immunoglobulin Fc receptors which include Fc receptors for IgG (FcγRs) and complement receptors CR1, CR3, CR4. These receptors allow recognition of encapsulated bacteria, subsequently the neutrophil may extend pseudopods all around the opsonised bacteria, engulfing it and forming a new organelle called a phagosome.

Two types of phagocytosis have been described, depending on the opsonins that are bound to the particle: Type I phagocytosis is characterised by pseudopodia extension, is mediated by FcγRs and requires the two Rho family Guanosine triphosphatases (GTPases): Rac, Cdc42. Type I phagocytosis induces an inflammatory response whereas type II phagocytosis, which is CR3-mediated, occurs through sinking of the C3bi-coated particle into the cell, requires only RhoA but not Rac and Cdc42 and does not induce an inflammatory response. This second type might be relevant to phagocytosis of apoptotic cells (Caron and Hall, 1998; Fadok *et al.*, 1998).

Recently, phagocytosis through CR3 of opsonised and non-opsonised zymosan was compared; under non-opsonic conditions zymosan was phagocytosed in a type I manner, whereas serum-opsonised zymosan induced a type II phagocytosis by human neutrophils and macrophages. Since these cells do not express the mannose

receptor it was assumed that internalisation was performed through CR3. However, the action of other receptors involved in the recognition of zymosan such as TLR2 and β-glucan receptor was not ruled out (Le Cabec *et al.*, 2002). Thus, CR3 may be able to mediate both types of phagocytosis through different mechanisms.

Probably, the more important factor related to the receptor that was used for phagocytosis is the type of signal that is induced in the neutrophil. The fact that phagocytosis by FcγRs generates a different outcome when compare to phagocytosis through CR3 suggests different signal molecules are induced in the neutrophil and in the past decade it has become clear that this is the case. This topic will be discussed in section 1.2.4.

1.2.3 Respiratory burst

One of the more remarkable functions of neutrophils is the production of radicals derived from oxygen which include superoxide, peroxide hydrogen, hydroxyl radicals and singlet oxygen by the action of the enzymatic complex NADPH oxidase. This enzyme is not only present in neutrophils but also in eosinophils, macrophages and B lymphocytes where the function is not well clarified. Because of the high consumption of oxygen this chain of events is also known as the respiratory burst. A variety of stimuli that activate neutrophils are able to induce the respiratory burst, including complement components such as C5a, PAF, IL-8, IFNγ, fMLP, PMA and phagocytosis.

NADPH is composed of five components: the membrane bound cytochrome b_{558}; the cytosolic factors $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the GTPase Rac2. Cytochrome b_{558} itself is a complex of two subunits $gp91^{phox}$ and $p22^{phox}$. Upon cell activation, the cytosolic components move to the membrane to form an electron-transfer system. The electrons are carried by the cytochrome b_{558} from NADPH in the cytosol, across the membrane, and delivered to oxygen present in an intracellular compartment such as a phagosome or in the extracellular milieu (Dahlgren and Karlsson, 1999).

Chronic granulomatous disease (CGD), a primary immunodeficiency where NADPH oxidase is defective is characterised by recurrent fungal (e.g. *Aspergillus fumigatus*) and bacterial (e.g. *Staphylococcus aureus*) infections. The cause is a defective gene in the $gp91^{phox}$ (two thirds of cases and linked to the X-chromosome) or in $p22^{phox}$ or other components (autosomic recessive). Studies in those patients affected by this disease have helped to understand the composition and actions of NADPH oxidase (Geiszt *et al.*, 2001).

NADPH oxidase catalyses the following reaction that leads to the production of superoxide anion(O_2^-): $2O_2 + NADPH \rightarrow 2O_2^- + NADPH^+ + H^+$

Superoxide dismutase (SOD) is useful to degrade O_2^- especially at low concentrations. At higher concentrations dismutation may happen spontaneously, through the catalysis of the following reaction that leads to the synthesis of peroxide hydrogen (H_2O_2): $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$

Peroxide hydrogen can reduce metals such as iron or copper to form hydroxyl radical ($OH^\cdot$), which is one of the most reactive radicals produced during the respiratory burst, the reaction that occurs to form hydroxyl is: $H_2O_2 + Fe^{2+} \rightarrow OH^\cdot + OH^- + Fe^{3+}$

Myeloperoxidase, which is a heme enzyme that is characteristic of azurophilic granules, converts H_2O_2 into a more potent toxic agent through the following reaction: $Cl^- + H_2O_2 \rightarrow OCl^- + H_2O$

In a further reaction singlet oxygen radicals ($^1O_2$) are formed. They have been attributed to the damage that neutrophils may cause to the normal tissue when the respiratory burst occurs: $H_2O_2 + OCl^- \rightarrow {}^1O_2 + H_2O + Cl^-$

Two different reactions help to decrease the amount of peroxide hydrogen produced. These reactions, which are very important as anti-oxidative pathways and limit further damage to normal tissue (Forman and Torres, 2002), are:

$H_2O_2 \rightarrow H_2O + O_2$ mediated by catalase (present in peroxisomes)

H_2O_2 + Glutathione (2GSH) → $2H_2O$ + (Glutathione disulfide)GSSG
(this reaction is catalysed by Glutathione peroxidase).

The respiratory burst participates in killing micro-organisms that have been phagocytosed. Initially it was accepted that oxidative changes in different structures and enzymes of the microorganism was enough to induce killing. However, this was challenged recently, when in a mouse model that lacks proteases but with proper NADPH oxidase activity, killing was affected. The explanation for this lies in the ability of O_2^- to induce potassium influx into the cells and a rise in the pH important for the activity of proteases (Reeves *et al.*, 2002).

Different ways have been used to determine the respiratory burst in neutrophils; some of the common ones are described in Table 1.2.

Table 1.2 Techniques commonly used for measuring oxygen radicals at intra or extracellular level (adapted from (Dahlgren and Karlsson, 1999))

Measuring principle	**Technique**	**Metabolite detected**
SOD- inhibitable reduction of cytochrome c	Photometry	Superoxide
Peroxidase-dependent oxidation of scopoletin	Fluorometry	Peroxide hydrogen
Peroxide-dependent isoluminol-amplified chemiluminescence	Luminometry	Superoxide
NBT reduction	Precipitation reaction	Superoxide(?)
Peroxidase-dependent luminal amplified chemiluminescence	Luminometry	Superoxide (?)
Oxidation of DHR123	FACS	Peroxide hydrogen

1.2.4 FcγRs

FcγRs are members of the immunoglobulin superfamily which contain two or three extracellular domains. Three different classes have been described: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16).

FcγRI is a 72kDa glycoprotein, comprising a 55kDa protein core and a number of N-linked glycosylation modifications potentially at positions 59, 78, 152, 159, 163, 195 and 240. FcγRI is often referred to as the high affinity IgG receptor which is capable of binding monomeric IgG by its Fc region. It is encoded by three different genes: A, B and C, all located in humans on chromosome 1q21. Of the three genes, only A encodes an integral receptor, B and C contain stop codons that might translate a soluble form of the receptor.

FcγRI has three extracellular domains (Figure 1.2) and is expressed in monocytes, macrophages, eosinophils, mast cells and dendritic cells (DCs). FcγRI is poorly expressed on resting neutrophils, but expression is increased upon activation of the neutrophil (Shen *et al.*, 1987). Cytokines able to induce the expression of FcγRI include IFNγ, IL-10 and G-CSF, whereas treatment of neutrophils with IL-4 decreases it (Hulett and Hogarth, 1994). FcγRI, the high affinity IgG receptor, binds to monomeric IgG through the third domain and to CRP, in the presence of calcium, with high affinity (0.81×10^{-9}M) (Bodman-Smith *et al.*, 2002)

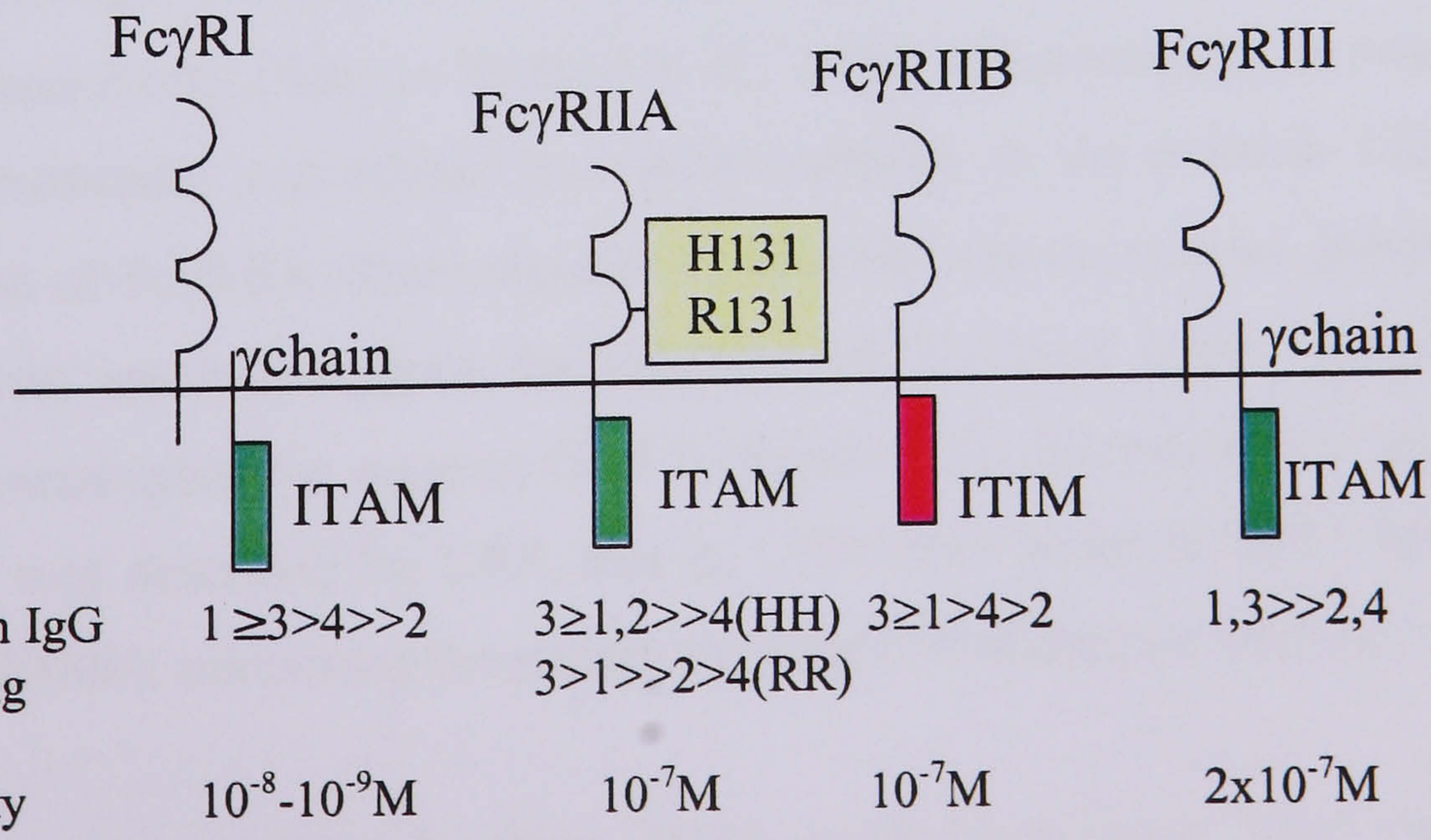


Figure 1.2 FcγRs Structure and binding to human IgG.

Murine FcγRI (mFcγRI) has the same structure as the human protein, although its ability to bind IgG is different from humans since it has a higher affinity for IgG2a, Kd = 10^{-7} – 10^{-8}M, than all the other subclasses. It is well expressed on monocytes, macrophages and neutrophils (Hulett and Hogarth, 1994). No crystal structure or functional FcγRI (or other FcγRs) has yet been expressed in a recombinant form in usable amounts.

Human FcγRII has three different genes: FcγRIIA, FcγRIIB and FcγRIIC, all located in chromosome 1q23-24. FcγRIIA is a 40kDa glycoprotein, with a protein core of 36 kDa. There are two potential N-glycosylation sites in positions 97(Asn) and 178(Asn). It is expressed on NK cells, DCs, Langerhans cells (LCs), macrophages, eosinophils, basophils, platelets and neutrophils (Takai, 2002; Kepley *et al.*, 2000). IL-4 has been shown to decrease the expression of FcγRIIA (Hulett and Hogarth, 1994) whereas IL-4 increases the expression of FcγRIIB on monocytes (Tridandapani *et al.*, 2002a).

FcγRIIA has been crystallised and its structure determined, revealing the presence of several loops in the second domain of the receptor which are important for binding to IgG1 and IgG2 which bind to different regions of the domain (Maxwell *et al.*, 1999).

Initially, it was reported that some individuals bound better to murine IgG1 (mIgG1) than others through FcγRIIA, they were described as high responders and low responders respectively (Van de Winkel *et al.*, 1987). Subsequently, it was shown that this characteristic was related to a polymorphism in the position 131 of the second domain of FcγRIIA where arginine or histidine can be present. Receptors of individuals who are homozygous for histidine (H131) bind better to IgG2 than individuals homozygous for arginine (R131) (Figure 1.2) (Warmerdam *et al.*, 1991). The opposite was described for CRP, that is, CRP binds better to R131 than H131 (Stein *et al.*, 2000b), mimicking the pattern for mIgG1 binding (see section 1.3.2.2).

In the same way, neutrophils from H131 individuals have been shown to phagocytose IgG2-opsonised group B type III Streptococcus strain M732 (Sanders *et al.*, 1995) and IgG2-opsonised *Neisseria meningitidis* (Fijen *et al.*, 2000) more

effectively than neutrophils from R131 individuals. This is thought to be the reason why R131 homozygous individuals are more frequently found to suffer recurrent pneumococcal infections (Sanders *et al.*, 1994).

FcγRIIB is a 40kDa glycoprotein, with a protein core of approximately 29 kDa. It has three different potential N-glycosylation (N-acetylglucosamine) sites:106 (Asn), 180(Asn) and 187(Asn). During transcription alternative splicing gives rise to two products RIIB1 and RIIB2. FcγRIIB is expressed by B-and T-lymphocytes, mast cells, basophils, eosinophils, DCs and LCs (Takai, 2002; Kepley *et al.*, 2000). Recently, FcγRIIB was shown to be expressed on monocytes in an IL-4 dependent way; previously mRNA for FcγRIIB was shown on neutrophils (Cassel *et al.*, 1993; Tridandapani *et al.*, 2002a).

FcγRIIC represents a cross-over between FcγRIIA and FcγRIIB, that is, the extracellular domains, transmembrane domain and the first intracellular domain are from IIB, the other two intracellular domains are from IIA (Warmerdam *et al.*, 1993). FcγRIIC is expressed on monocytes, neutrophils and four different transcripts on NK cells (Metes *et al.*, 1998).

Mice only express FcγRIIB and no FcγRIIA, which is more heterogeneous in size than in humans, with a molecular weight ranging from 40 to 60 kDa. The extracellular and transmembrane domains are highly conserved between humans and mice, but both species have isoforms that differ in the intracytoplasmic domains. The affinity for the murine IgG subclasses is the same ($<10^{-7}$ M) for IgG1, 2a and 2b and lower for IgG3. This receptor is expressed in monocytes, mast cells, macrophages, platelets, neutrophils and B cells (Hulett and Hogarth, 1994)

FcγRIII is also heterogeneous in size with a molecular weight range from 50 to 80 kDa. This heterogeneity is generated by N-linked glycosylation of both FcγRIIIA and FcγRIIIB with five: (positions 53, 63, 92, 180, 187) and six (positions 56, 63, 82, 92, 180, 187) potential residues respectively. The latter receptor is the only membrane FcγR that is not an integral part of the membrane but is attached by a glycosylphosphatidylinositol (GPI) moiety. FcγRIIIA is expressed on monocytes,

macrophages, NK cells, mast cells, eosinophils, DCs, LCs, and γ/δ T cells, whereas FcγRIIIB is found on neutrophils and eosinophils. TGFβ increases FcγRIIIA expression. IFNγ, GM-CSF and G-CSF induce the expression of FcγRIIIB, on the other hand IL-4 decreases FcγRIIIA and TNFα decreases FcγRIIIB expression on neutrophils; soluble FcγRIII has been found in the serum of infected patients as a consequence of cleavage from neutrophils by serine proteases (Khayat *et al.*, 1987).

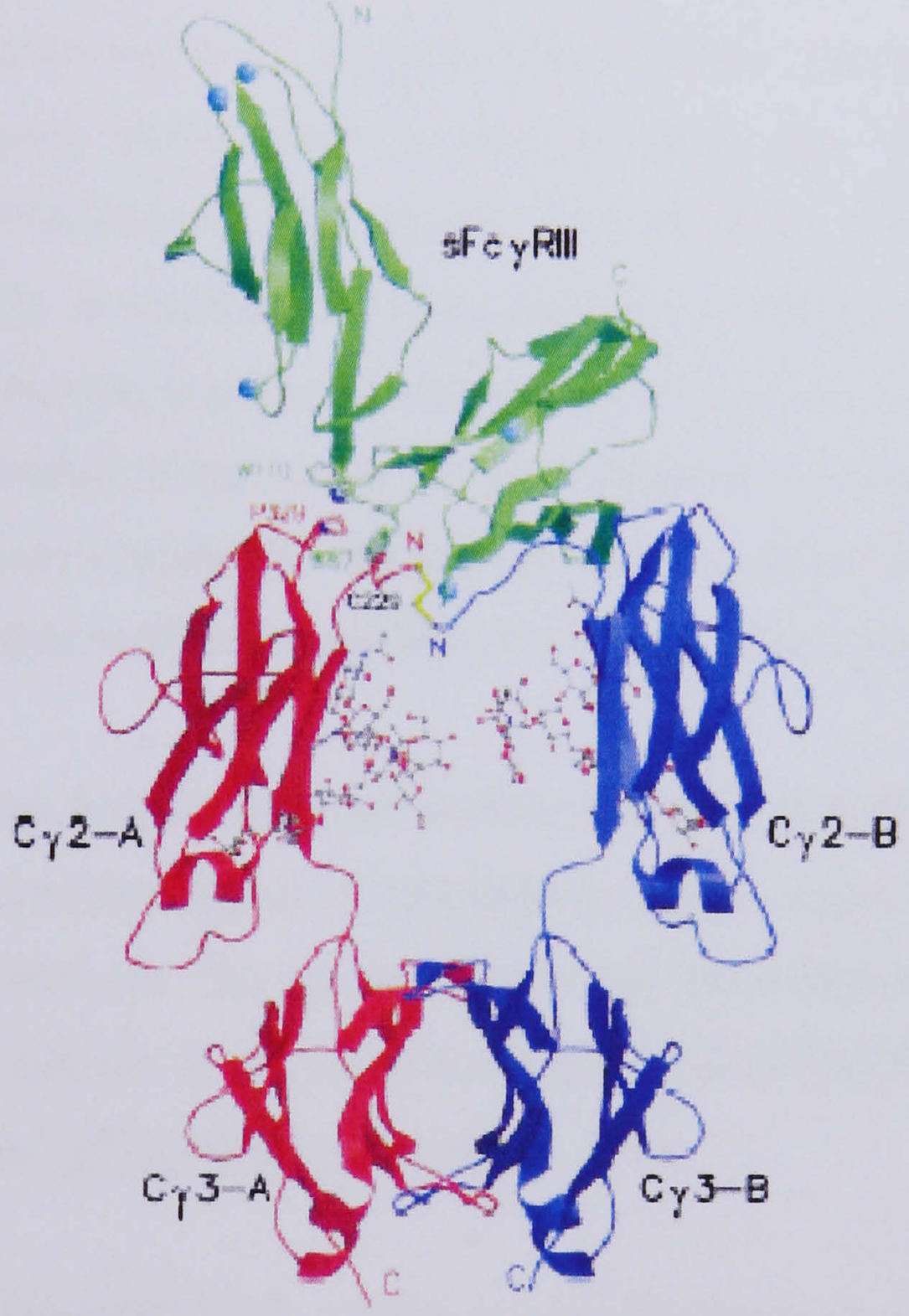


Figure 1.3 The overall structure of the sFcγRIII- human Fc1 complex. The crystal structure of the soluble FcγRIII with the monoclonal human Fc fragment of IgG1(hFc1) is shown. Stereo ribbon representation with the dimer axis of hFc1 (red and blue) orientated vertically. The 'proline sandwich' consisting of Pro 329 of the Cγ2-A domain and Trp 87 and Trp 110 of sFcγRIII (green) is shown in ball and stick fashion together with the carbohydrate residues of the Fc fragment and the interchain disulphide bridge (yellow) of the Cys 229 residues. The potential N-glycosylation sites in sFcγRIII are shown as cyan balls. Taken from Sonderman *et al.*, (2000).

X-ray crystal structures of FcγRIII with IgG have also been reported (Figure 1.3). Soluble FcγRIIIB bound to the second domain of the Fc portion of the IgG1

molecule by its second domain, ensuring a 1:1 stoichiometry of the complex (Sondermann P *et al.*, 2000).

Two allelic forms of FcγRIIIB have been described and are designated as neutrophil-specific antigen (NA) NA1 and NA2 which differ by five nucleotides and four amino acids, with NA2 containing two additional N-linked glycosylation sites (six versus four). Neutrophils from individuals homozygous for NA1 exhibit better phagocytosis of IgG1-opsonised *Staphylococcus aureus*, *Haemophilus influenzae* type b and *Neisseria meningitidis* as well as IgG3 anti Rhesus D-opsonised erythrocytes than NA2-homozygous donors (Bredius *et al.*, 1994). There are also changes in FcγRIIIA at position 48 which generates 3 different allotypes with the presence of either leucine, arginine or histidine (de Haas *et al.*, 1996) and at position 158 which may contain either valine or phenylalanine and may have functional consequences because it alters IgG binding and has been associated with an elevated risk for systemic lupus erythematosus (Deo *et al.*, 1997; Koene *et al.*, 1998).

In contrast to human FcγRIII only one isoform has been described in mice for this receptor. The extracellular region of mFcγRIII is highly conserved with mFcγRIIb (95% amino acid identity). The transmembrane and intracellular domains are more similar to human FcγRIIIA. It has the same affinity ($Kd<10^{-7}M$) for IgG1, 2a and 2b, but lower affinity for IgG3 (Hulett and Hogarth, 1994).

The main human neutrophil FcγRs, which are constitutively expressed, are FcγRIIA and FcγRIIIB. Decreased levels of FcγRIIIB are usually found on neutrophils spontaneously suffering apoptosis (Homburg *et al.*, 1995). FcγRI is inducible on normal neutrophils and may reach up to 15000 receptors per cell after the cells are cultured in the presence of 100IU IFNγ. The same treatment did not alter expression of FcγRIIA (about 25000/cell) or FcγRIIIB (about 200000 molecules per cell) as analysed by FACS (Hoffmeyer *et al.*, 1997).

FcγRI and FcγRIII in both human and mouse co-ligate with a γ-chain that contains an immunoreceptor tyrosine-based activation motif (ITAM), whereas FcγRIIA contains an ITAM in its cytoplasmic domain. It has been shown that there is co-operation

between the FcγRs expressed on neutrophils as has been shown for FcγRIIIB which enhances phagocytosis when it is cross-linked with FcγRIIA (Salmon *et al.*, 1995). More recently FcγRI has been shown to be able to signal through FcγRIIA in U937 cells differentiated to a macrophage-like phenotype with dibutyryl cAMP (Cameron *et al.*, 2001). Dr Bodman-Smith in our laboratory has shown that this can lead to phagocytosis (unpublished data).

On the other hand, FcγRIIB in humans and mice contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) which down-regulates phagocytosis. Co-transfection of FcγRIIB was able to decrease phagocytosis of IgG-opsonised sheep red blood cells (SRBC) by Cos cells which were transfected with a chimeric receptor containing the extracellular portion of FcγRI and the intracellular domain of FcγRIIA. Co-transfection with a mutant RIIB lacking the intracellular domain did not inhibit phagocytosis, stressing the importance of the presence of ITIM (Hunter *et al.*, 1998).

In phagocytes, the initial ITAM phosphorylation is due to the action of the family of Src tyrosine- kinases which include Fgr, Fyn, Hck, Lyn, Yes and Src, which then phosphorylate the important tyrosine kinase Syk. In monocytes, Hck and Lyn have been associated with FcγRI, whereas only Hck has been associated with FcγRIIA (Gerber and Mosser, 2001). In neutrophils, Fgr has been found in association with FcγRIIA (Hamada *et al.*, 1993).

Tyrosine kinases remain inactive until there is interaction of a phosphorylated tyrosine with its own Src-homology domain (SH2) (Garcia-Garcia and Rosales, 2002). Receptor cross-linking allows further phosphorylation of tyrosines located nearby (Figure 1.3). Phosphorylated ITAMs become a docking site for the SH2 domains of Syk. Syk is important in transmitting the phagocytic and respiratory burst signal after engagement of ITAM-containing Fcγ receptors (Kiefer *et al.*, 1998; Greenberg, 1999b).

Transfected Cos-7 cells have been widely used to analyse the effect of different FcγRs in the wild type form or mutated in different regions as has been the case for FcγRIIA or mutations on the γ-chain for studies on FcγRI and IIIB. These cells can be studied with or without co-transfection with Syk. Syk was shown to increase phagocytosis of IgG- opsonised particles when present with an appropriate ITAM containing receptor (Indik *et al.*, 1995).

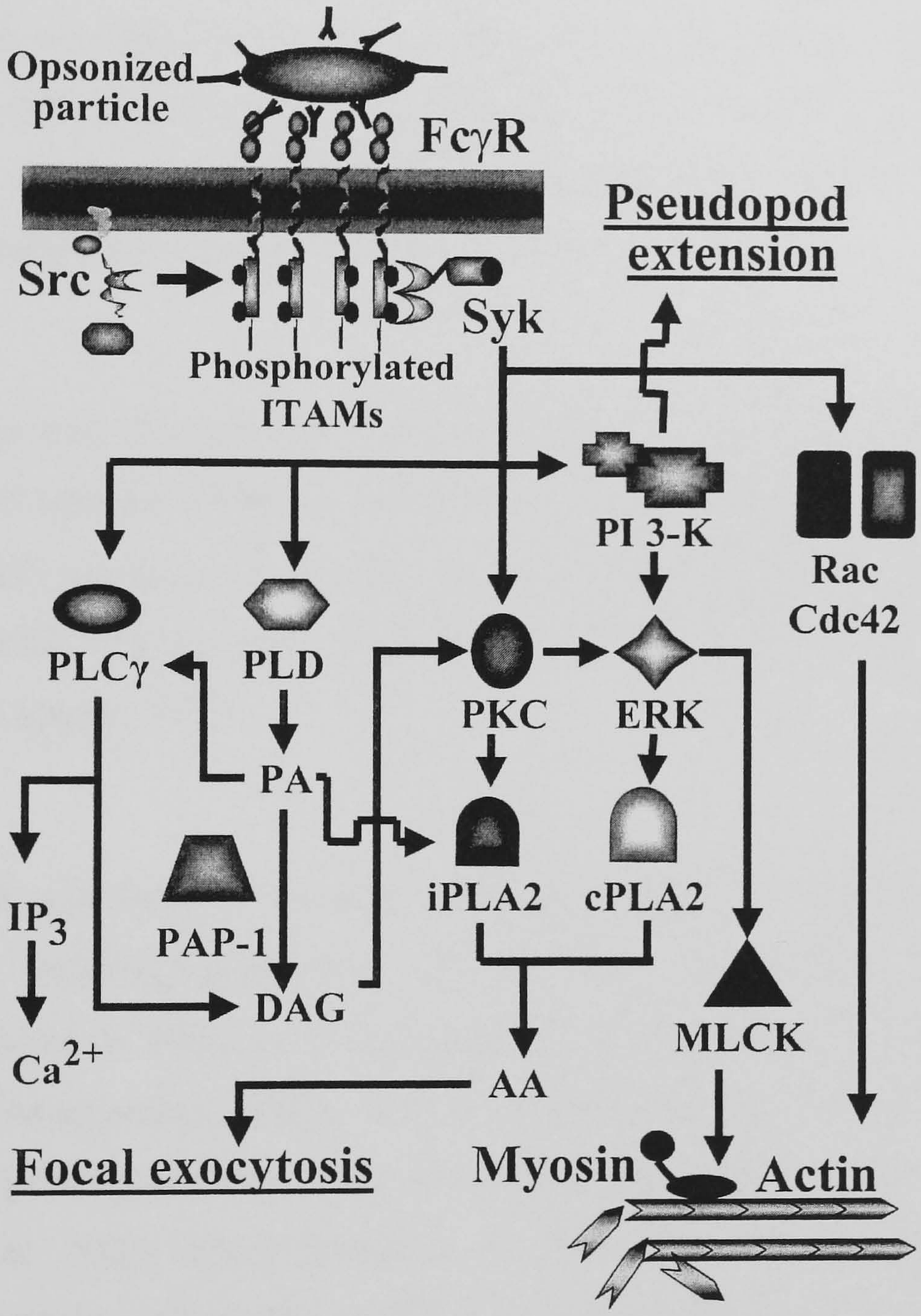


Figure 1.4 FcγR-mediated signalling to provoke phagocytosis. AA: Arachidonic acid; DAG: diacylglycerol; ERK: extracellular signal-regulated kinase; IP_3: inositol 1,4,5-trisphosphate; ITAM: immunoreceptor tyrosine-based activation motif; MLCK: myosin light chain kinase; PAP-1: phosphatidic acid-phosphatase-1; PI 3-K: Phosphoinositol-3 kinase; PKC: protein kinase C; iPLA2: Inducible phospholipase A2; cPLA2: constitutive phospholipase A2; PLCγ: Phospholipase Cγ; PLD: Phospholipase D. Taken from Garcia-Garcia and Rosales, (2002)

The downstream pathways stimulated by active Syk involves calcium, Protein kinase C (PKC), phospholipase A2 (PLA_2), Phospholipase Cγ (PLCγ), Phospholipase D

(PLD), Phosphoinositol-3 kinase (PI-3K), GTPases of the Rho family and the mitogen-activated protein kinases (MAPKs) (Figure 1.4).

Once phosphorylated, MAPKs phosphorylate specific serine and threonines of substrates including protein kinases, phospholipases, transcription factors and cytoskeletal proteins. MAPKs serve as phosphorylation substrates for MAPK kinases (MKKs) which are highly selective in phosphorylating specific MAPKs. MAPK kinase kinases (MKKKs) are the third component of the phosphorelase system which phosphorylate and activate specific MKKs. Scaffolding proteins organise their interaction (Johnson and Lapadat, 2002).

There are three well characterised pathways of MAPKs including the extracellular signal-regulated kinases (ERK1 and ERK2), the c-Jun NH2-terminal kinases (JNK1 JNK2 and JNK3) and four p38 enzymes ($\alpha,\beta,\gamma,\delta$). Other MAPK pathways may exist as ERK5 and ERK7 have recently been identified but still are not well characterised (Johnson and Lapadat, 2002).

Both FcγRIIIB and FcγRIIA mediate biological activities such as degranulation, phagocytosis, Antibody-dependent cell-mediated cytotoxicity (ADCC) and superoxide generation. Phagocytosis is mediated by both receptors after activation of MAPKs and subsequently ERK2, which links the MAP kinase pathway to the activation of cytoskeletal components through phosphorylation of myosin light chain (Mansfield *et al.*, 2000). The p38 MAP kinase pathway is important for chemotaxis, superoxide generation and synthesis of IL-8 by neutrophils (Zu *et al.*, 1998).

Although early reports have shown that clustering of FcγRs is necessary to increase the concentration of intracellular calcium of human neutrophils, a more recent study shows that intracellular increase of the concentration of calcium is a late event that occurs after phagolysosome fusion (Worth *et al.*, 2003). When an antibody IV.3, which binds all FcγRII receptors, was used to activate human neutrophils through FcγRIIA, levels of calcium were lower when compared to levels reached when both

FcγRIIA and FcγRIII were targetted using both IV.3 and an antibody that binds FcγRIII (3G8 F(ab')$_2$) or when cells were stimulated at the same time using immune complexes (Hundt and Schmidt, 1992). FcγRIIA has the L-T-L motif that participates in the calcium mobilisation within the cell that has been described as a "wave propagation of calcium" (Worth *et al.*, 2003), thus looking at the phagolysosomal formation in conjunction with mobilisation of calcium would be a more physiological way to study the role of FcγRs on neutrophil function.

Cross-linking of FcγRIIIB alone using 3G8 F(ab')$_2$ in neutrophils stimulates an increase of intracellular calcium, activates actin polymerization, respiratory burst activity, degranulation and phagocytosis. These activities are further amplified when there is cross-linking with CR3 or with FcγRIIA (Ohkuro *et al.*, 1995). Both receptors activate MAPKs with different efficacy; both stimulate ERK which is necessary for actin polymerization, but act as negative regulators of respiratory burst activity. Activation of p38 is necessary for respiratory burst activity stimulated by FcγRIIIB but not by FcγRIIA (Coxon *et al.*, 2000).

Mechanisms of control of the signal transduction in neutrophils induced by cross-linking FcγRs or other molecules exist and involve different regulatory molecules such as CD45, CD148 and CD47. CD45 is a protein tyrosine phosphatase (PTP) with two PTP domains. CD148 (also known as human PTP or DEP-1 (Density enhanced protein tyrosine phosphatase-1)) contains only one PTP domain. Both CD45 and CD148 are able to decrease FcγRIIA mediated effects in neutrophils. These molecules interfere with the phosphorylation of Syk and PLCγ induced by FcγRIIA and therefore alter the respiratory burst, and the increment in the intracellular concentration of calcium (Hundt and Schmidt, 1997).

CD47 expressed on neutrophils is able to control transmigration and chemotaxis of these cells upon interaction with signal regulatory protein (SIRPα) which is expressed on monocytes, DCs, and also on neutrophils. SIRPα contains an ITIM domain transducing a regulatory signal into the cell, although SIRPα-independent

regulatory signals have been obtained upon interaction with thrombospondin, probably through PI-3K or tyrosine kinases (Liu *et al.*, 2002).

The better known ITIM-containing molecule with ability to down-regulate cell function is FcγRIIB. The ITIM motif binds and activates the SH2-containing inositol 5' phosphatase (SHIP). The SH2 domain present in SHIP also associates with phosphorylated Shc a proline-rich protein that constitutively associates with Grb2 (an adapter protein). SHIP hydrolyses phosphatidylinositol -3,4,5 triphosphate, a product of PI-3K (Damen *et al.*, 1996; Kavanaugh *et al.*, 1996). This down-regulates activation of neutrophils and monocytes.

To our knowledge only expression of mRNA for FcγRIIB has been shown in neutrophils (Cassel *et al.*, 1993). Since both FcγRIIA and RIIB have identical extracellular domains it is difficult to show expression of RIIB on the surface of phagocytic cells. Direct activation of SHIP by FcγRI and RIIA has been suggested (Tridandapani *et al.*, 2002b; Cameron and Allen, 1999) and the presence of other receptors on neutrophils that contain ITIM might also be mechanisms by which phagocytosis or other activities induced by FcγRs on neutrophils are regulated.

1.2.5 Apoptosis of neutrophils

Apoptosis is a word derived from the Greek used to describe the process of leaves falling from tree or petals from flowers. It describes cells that shrink and fall away from tissues with little damage to the main organism. Apoptosis has been shown not only in mammalian cells but also in birds, amphibians, and insects, as a development process as well as one that controls the number of immune cells (Martin, 1998).

An apoptotic cell characteristically condenses its nucleus and cytoplasm as a result of dehydration, the staining intensity of chromatin increases because of condensation, there is marked blebbing of the plasma membrane and in the final stages, the cell

collapses into multiple fragments (apoptotic bodies) that are taken up by phagocytes. This process affects cells individually and does not lead to an inflammatory response (Martin, 1998). On the contrary the process of necrosis involves different events, the nucleus typically swells to occupy most of the cytoplasm, the staining intensity of chromatin decreases, the content of the cytoplasm is liberated to the extracellular medium, and this affects groups of contiguous cells. Necrosis promotes a strong inflammatory response (Martin, 1998).

One of the hallmarks of apoptosis is DNA cleavage which occurs randomly in necrotic cells whereas in apoptotic cells there is generation of more homogeneous DNA fragments. In most cells, two stages of DNA degradation are observed: initially, DNA is cleaved at the sites of attachment of chromatin loops to the nuclear matrix, which results in 50- to 300 kilobase size fragments. Later, DNA is cleaved at the internucleosomal sections, giving DNA fragments of approximately 180bp (Wyllie, 1980; Cohen and Duke, 1984). The enzyme caspase-activated deoxyribonuclease (CAD) is involved in the production of nucleosomal DNA fragments. Other enzymes such as endonuclease G also contribute to the cleavage of DNA, especially for the bigger fragments (Samejima *et al.*, 2001) .

Earlier events are described in apoptotic cells, one of them is the alteration of phospholipid expression in the outer leaflet of the plasma membrane which changes from choline phospholipids such as phosphatidylcholine and sphingomyelin to aminophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (Schlegel and Williamson, 2001).

Seigneuret and Devaux showed that an analog of PS introduced into the outer leaflet of erythrocytes was transported to the inner leaflet in an ATP-dependent way. This transport activity was termed the aminophospholipid translocase (Seigneuret and Devaux, 1984). Increased concentrations of calcium inactivate the translocase, and activate a non-specific, bidirectional lipid flipsite, distinct from the translocase called scramblase, which brings PS to the surface (Schlegel and Williamson, 2001).

PS contributes to the recognition of apoptotic cells as was shown on apoptotic lymphocytes that were phagocytosed by macrophages, (Fadok *et al.*, 1992) whose expression of a PS receptor: a class B scavenger receptor type I (SR-B1) has been demonstrated (Figure 1.5) (Shiratsuchi *et al.*, 1999). Integrins also are involved in the recognition of PS expressed on apoptotic neutrophils; for instance the receptor complex αvβ3/CD36 on macrophages binds to thrombospondin which binds to PS (Figure 1.5). Recently, it has been shown that protein S, which participates in the coagulation cascade as an anticoagulant, binds to PS and promotes phagocytosis of apoptotic cells by macrophages (Anderson *et al.*, 2003).

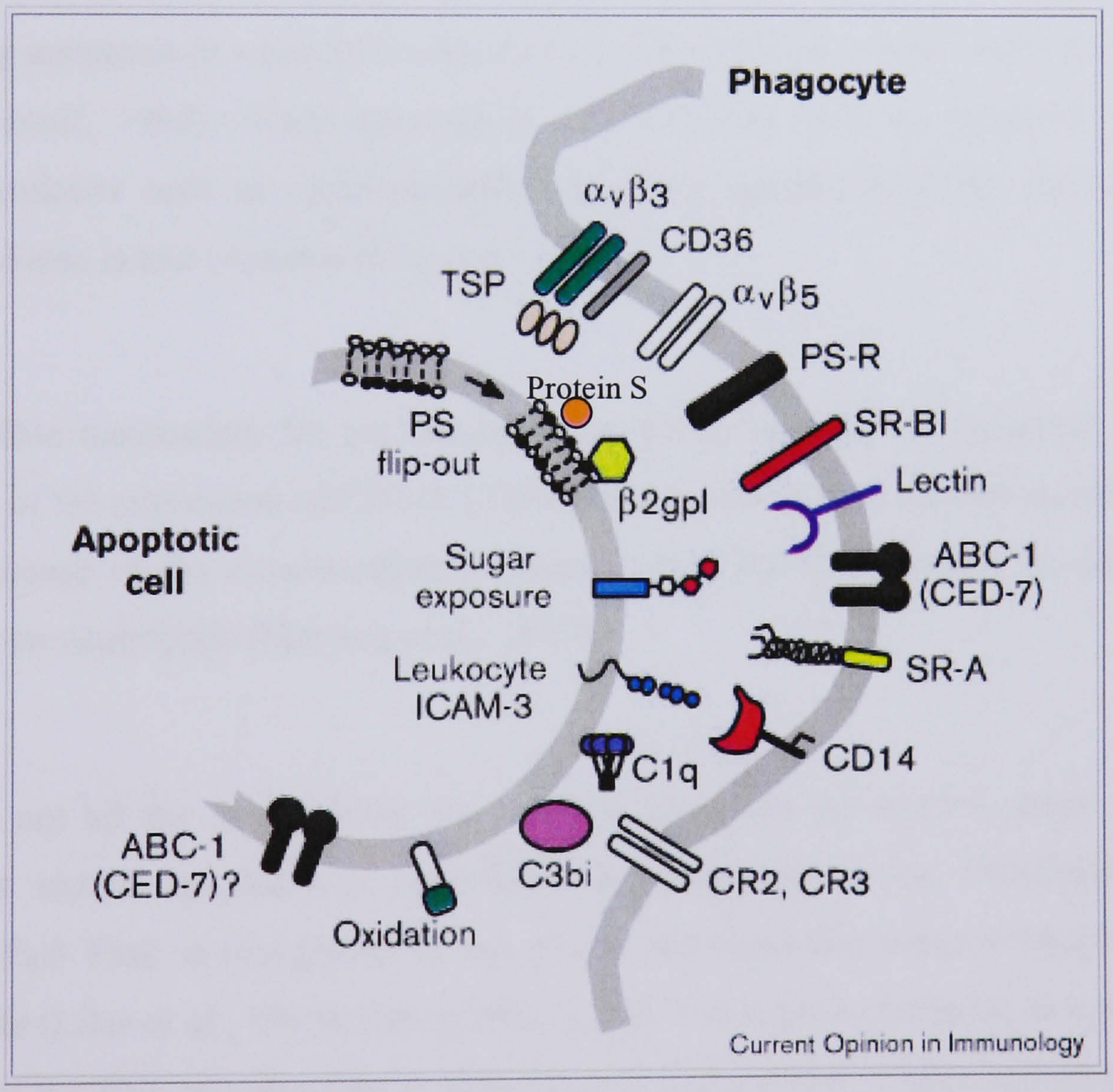


Figure 1.5 Some molecules involved in recognition and phagocytosis of apoptotic cells. ABC-1: ATP-binding cassette transporter 1; β2gpI: β2 glycoprotein I; PS: Phosphatidyl serine SR: Scavenger receptor; TSP: thrombospondin. Modified from Gregory (2000).

Another early event is a decrease in mitochondrial transmembrane potential, which is reflected by a loss of the ability of the cell to accumulate certain dyes in the

mitochondria such as rhodamine, this event is associated with a leakage of cytochrome c from the mitochondria to the cytoplasm, increased production of superoxide anion and increased content of the reduced form of cellular glutathione (Darzynkiewicz and Bedner, 2000). The enzymes responsible for these events described in apoptotic cells are the result of the activation of the caspase (cysteine-dependent aspartate –specific proteases) cascade (Cohen, 1997). See below and Figure 1.6.

The rapid turnover of neutrophils ends with apoptosis of the cell and non-inflammatory uptake by macrophages. Some cytokines are very important in preventing apoptosis in neutrophils and they include IFNγ, IL-2 and GM-CSF (Liles and Klebanoff, 1995). When neutrophils are incubated with an inhibitor of the protein synthesis such as cycloheximide, protection against apoptosis induced by these cytokines is lost (van den Berg *et al.*, 2001).

One possible mechanism for prevention of apoptosis induced by cytokines is the induction of the expression of CD11b/CD18 (Mac-1) which after ligation generates a minor increase of the concentration of calcium that has been shown to decrease apoptosis on neutrophils (Fanning *et al.*, 1999).

Although not all the mechanisms that regulate apoptosis are known, some of the ways that induce apoptosis in neutrophils are beginning to be elucidated. The complex Fas- FasL is recognised as one of the pathways that induces apoptosis in neutrophils (Liles *et al.*, 1996). Fas (CD90), a type I membrane receptor, is a 45-kDa member of the TNF receptor family which also includes CD40, CD27, CD30 and the receptor for lymphotoxin-β. Fas is expressed on various cells such as lymphocytes, monocytes-macrophages, eosinophils and neutrophils (Fanning *et al.*, 1999).

FasL is a type II membrane receptor, that is, its N-terminus is in the cytoplasm and its C-terminal region extends into the extracellular space. FasL is a 40kDa protein member of the TNF family of proteins that includes TNFα, lymphotoxin, TNF-

related apoptosis inducing ligand (TRAIL), CD40L and CD30L. FasL is predominantly expressed on activated T- and NK-cells, whereas its expression is constitutive on neutrophils (Nagata, 1997).

It has been shown that T-cells secrete a soluble form of FasL that can induce apoptosis in surrounding cells (Tanaka *et al.*, 1995); this soluble form is produced by the action of a metalloproteinase which also has the ability to induce soluble forms of TNFα and CD40L (Tanaka *et al.*, 1996). Moreover, FasL which also can be secreted by neutrophils was found to be chemotactic for neutrophils (Ottonello *et al.*, 1999). FasL can induce apoptosis in neighbouring neutrophils in a way to control the inflammatory response.

Bacterial ingestion may induce neutrophil apoptosis via an oxygen dependent mechanism (Watson *et al.*, 1996). Neutrophils from CGD patients have been allowed to phagocytose heat-killed *Staphylococcus aureus*, apoptosis was analysed and compared with neutrophils from normal donors. Phagocytosis of bacteria induced apoptosis in both groups of neutrophils, but neutrophils from CGD patients showed reduced apoptosis analysed by Annexin V, which suggests that oxygen radicals have a role in neutrophil apoptosis (Yamamoto *et al.*, 2002). Furthermore, antioxidants such as taurine, N-acetylcysteine and glutathione also decrease Fas-mediated apoptosis (Fanning *et al.*, 1999). On the other hand, members of the Bcl-2 family protein regulate apoptosis in neutrophils (Iwai *et al.*, 1994; Liles and Klebanoff, 1995).

Complexes of Fas occur in the plasma membrane spontaneously, but upon binding of FasL, a more stable structure forms, called death-inducing signalling complex (DISC). The Fas DISC contains the adaptor protein Fas-associated death domain protein (FADD) and caspases 8 and 10, which can initiate the process of apoptosis (Figure 1.6). In some cells, caspase-8 is sufficient to directly activate other members of the caspase family. In other cells, proper activation of effector caspases by Fas depends on an amplification loop that depends on the activation of caspases 3 and 9 which in turn activate caspase 8 (Wajant, 2002).

TNFα appears to have a divergent effect; at low concentrations (0.1 – 1 ng/ml) survival of neutrophils increases, whereas at higher concentrations (10 – 100 ng/ml) via binding to the type 1 TNFR (TNF-R1), accelerates neutrophil apoptosis at early time points (van den Berg *et al.*, 2001). After 6 hours 4.6% of neutrophils at 5 x 10^6/ml were apoptotic, whereas in the presence of 25ng/ml TNFα this had increased to 17.2%. In contrast at 20 hours the same amount of neutrophils in the presence of the same concentration of TNFα displayed 46.9% apoptosis compared to 55.9% suffered by neutrophils alone (Murray *et al.*, 1997). Induction of apoptosis by TNFα can be increased by the addition of gliotoxin, an inhibitor of NFκB (Ward *et al.*, 1999).

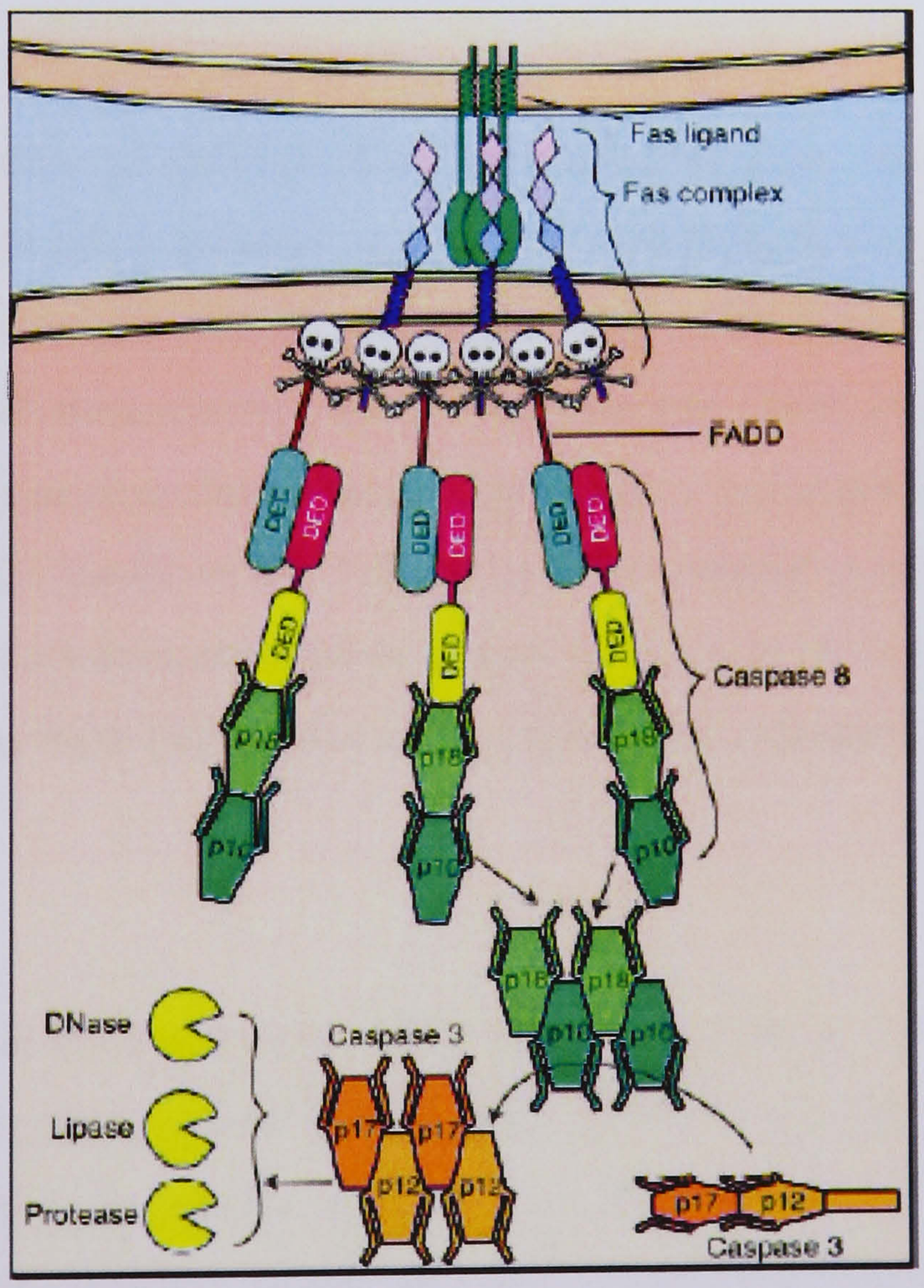


Figure 1.6 FasL-induced apoptosis occurs by autoproteolytic processing of caspase-8. Note that caspase 8 is composed of three subunits (DED, p18 and p10). Taken from Wajant (2002) DED: Death effector domain; FADD: fas- associated death domain protein,

Recently, TRAIL has been shown to induce apoptosis of neutrophils after ligation of TRAIL-R2 rather than TRAIL-R1. This apoptotic phenomenon was not dependent on NF-κB but was inhibited by addition of GM-CSF (Renshaw *et al.*, 2003).

In order to determine apoptosis in a cell, frequently used assays are based on the concentration of particular caspases, expression of PS in the membrane of the cell, or the extension of DNA fragmentation. The concentration of Caspases can be determined by ELISA, expression of PS by binding to Annexin V conjugated to a fluorescent compound like FITC, and DNA fragmentation by electrophoresis which shows the formation of DNA "ladders". Alternatively, fragmented DNA ends can be detected by attachment of modified nucleotides that can be detected by FACS or by confocal microscopy (Darzynkiewicz and Bedner, 2000).

The majority of studies so far have been performed *in vitro*. Apoptosis *in vivo* is more difficult to visualise because apoptotic neutrophils, once phagocytosed by macrophages, are destroyed within the next 20 minutes. In 1992, it was shown that macrophages isolated from synovial fluid from inflamed joints of patients suffering acute sterile arthritis, preferentially phagocytosed ageing neutrophils rather than non-apoptotic neutrophils (Savill *et al.*, 1989). Later by electron microscopy apoptotic neutrophils obtained by bronchoalveolar lavage from lungs of neonatal babies with airway inflammation were also shown to be ingested by macrophages (Grigg *et al.*, 1991).

Little is known about the mechanisms that control apoptosis *in vivo*. IL-10 has been shown to help in the resolution of inflammatory airways in rats treated with LPS by clearing neutrophils more rapidly than the rate observed in untreated rats (Cox, 1996). More work is needed to clarify the role of different cytokines and molecular pathways involved in apoptosis by using models of inflammation in which specific genes have been targeted to study their function.

1.3 Acute phase proteins with innate immune function

The innate immune response can be defined as the ability to recognise a particular substance as foreign without previous contact with it. This capacity can be accomplished taking into account motifs that are commonly expressed in particular micro-organisms but not by our own cells, known as pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997).

The immune system has taken advantage of PAMPs and has created proteins and receptors that allow it to recognise a potential pathogen; these are called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000). PRRs are important members of the innate immune system which also involve the role of cells such as DCs, NK cells, macrophages and neutrophils and also make an important connection with the adaptive immune system.

Once a PRR has identified a foreign particle through its binding properties, a sequence of events amplifies the immune response in terms of quality and quantity and adjustments at endocrine, metabolic, haematopoietic levels occur. This chain of events is known as the acute phase response.

During the acute phase response the concentration of some proteins (acute phase proteins – APPs) increase (positive acute phase response) whereas others decrease (negative acute phase response) (Table 1.3). In the former group, there are proteins the concentration of which increase up to 1000 times and therefore are considered major APPs. This group includes CRP and acute phase- serum amyloid A (A-SAA). Others do not increase at the same proportion but can reach levels up to 3 times, e.g. mannose binding lectin (MBL), α1-acid glycoprotein (AGP), α1-antitrypsin (AAT) and complement proteins, among others, although these proteins represent a greater synthetic proportion of hepatocyte output (Table 1.3).

Many of these proteins are components of the innate immune system with ability to recognise PAMPs. Some have the capacity to opsonise micro-organisms or altered/damaged cells facilitating their removal and clearance by phagocytic cells.

For some APPs specific receptors are found to promote interaction of APPs with neutrophils, macrophages and dendritic cells. For example, MBL interacts with CR1, a complement receptor expressed by neutrophils and macrophages; CRP interacts with Fcγ receptors in phagocytic cells and activates complement. α_2 macroglobulin interacts with CD91 (the receptor for the heat shock protein gp96) to promote more efficient presentation (Binder *et al.*, 2001)

Table 1.3. Biochemical characteristics of some human acute-phase proteins

Protein	Molecular weight/ Gene Location	Normal values	Increase	Proposed function
CRP	118 kDa 1q21-23	1-10 μg/mL	Up to 1000 fold	Innate immunity
A-SAA	12.5 kDa 11p15.1	1-5 μg/mL	Up to 1000 fold	Metabolism Innate immunity
MBL	780 kDa 10q11.2-q21	1-20 μg/mL	Up to 3 fold	Innate immunity Lectin pathway
AGP	41 – 43 kDa 9q31-32	0.36-1.46 mg/mL	Up to 5 fold	Immunomodulation
AAT	60kDa 14q32.1	1.5 - 3.2mg/mL	Up to 4 fold	Serine proteinase inhibitor
Fibrinogen	23.9kDa 4q28	1.5 – 4 mg/mL	Up to 4 fold	Coagulation
Haptoglobin	36.8kDa 16q22.1	0.3 – 2 mg/mL	Up to 4 fold	Metabolism of Haemoglobin
C3	195kDa 19p3.2-3.3	ca. 1.3 mg/mL	50%	Complement Cascade
C4	206kDa	ca. 0.6 mg/mL	50%	Complement Cascade

AAT: α1-antitrypsin, AGP: α1-acid glycoprotein, A-SAA: acute –serum amyloid A, CRP: C-reactive protein, MBL: Mannose binding lectin

Table 1.4. Pattern Recognition Receptors (PRRs) and Pathogen- associated molecular patterns (PAMPs).

Protein Family	Soluble proteins	Receptor	PAMP
C-type lectins	MBL		Mannose, fucose, N-acetyl glucosamine, glucose
		Mannose receptor	Mannose, fucose, N-acetyl glucosamine, glucose
	Surfactant protein A		Preference for fucose
	Surfactant protein D		Preference for maltose
		DEC-205	
Leucine-rich proteins	Soluble CD14		LPS
		TLR2	LTA, zymosan and peptidoglycan
		TLR4	LPS, pneumolysin
		TLR5	Flagellin
		TLR9	CpG
		CD14	LBP-LPS
Pentraxins	CRP		Phosphorylcholine, phosphorylated galactose, lysophospholipids and phosphorylated polysaccharides
	SAP		Phosphoethanolamine, sulphated polysachharides
	Pentraxin 3		Galactans, fungi
Scavenger receptor		CLASS A	LPS, LTA
		CLASS B	Plasmodium infected erythocytes
		CLASS C	β-glucan
		MARCO	
Integrins		CR3, CR4	LPS

When two or more PRR bind to the same pathogen a synergistic response can be observed. As an illustration, Toll-like receptor 4 (TLR-4, Table 1.4) is able to bind to

lipopolysaccharide (LPS), and CD14 binds to lipopolysaccharide binding protein (LBP) that interacts with LPS. When these two receptors both recognise LPS on the surface of the same cell the activation of the cell is increased exponentially.

Upon binding, usually to more than one PAMP structure, and thereby facilitating high affinity interactions with the micro-organism, the phagocytic cell becomes activated and engulfs it. After processing and presentation of specific fragments (epitopes) to T cells, amplification of the immune response occurs. In this way, a connection between the innate and adaptive immune systems is accomplished; thus, APPs play an important role in this process. In addition to complement mediated effects, the mannose receptor has been shown to improve presentation by antigen presenting cells (Prigozy *et al.*, 1997).

A similar effect might be assumed to occur for other APPs and complementary receptors that have the capacity to bind to the same ligand and therefore reinforce the function of the cell. On the other hand, these interactions might be useful as immunomodulatory signals. For instance, some APPs instead of promoting a particular function may actually decrease it, in order to exert a regulatory effect over the immune response

1.3.1 Mannose Binding Lectin

MBL is a protein of the collectin family, which consists of a collagen-like region, a neck region and a lectin domain (Figure 1.7). The lectin is found at the C-terminal region and contains a calcium dependent carbohydrate recognition domain (CRD) on each subunit chain (Kawasaki *et al.*, 1978). Other members of the collectin family include surfactant protein A (SP-A) and surfactant protein D (SP-D).

MBL is synthesised by the liver, its functional form is a multimeric protein of up to six 96 kDa subunits, each consisting of 3 identical polypeptide chains of approximately 32 kDa. MBL recognises fucose, mannose and N-acetyl-glucosamine oligosaccharides present in a wide range of bacteria (e.g. *Salmonella montevideo*), viruses (e.g. Influenza virus), fungi (e.g. *Cryptococcus neoformans*) and parasites (e.g. *Leishmania major*) (Turner, 1996; Petersen *et al.*, 2001).

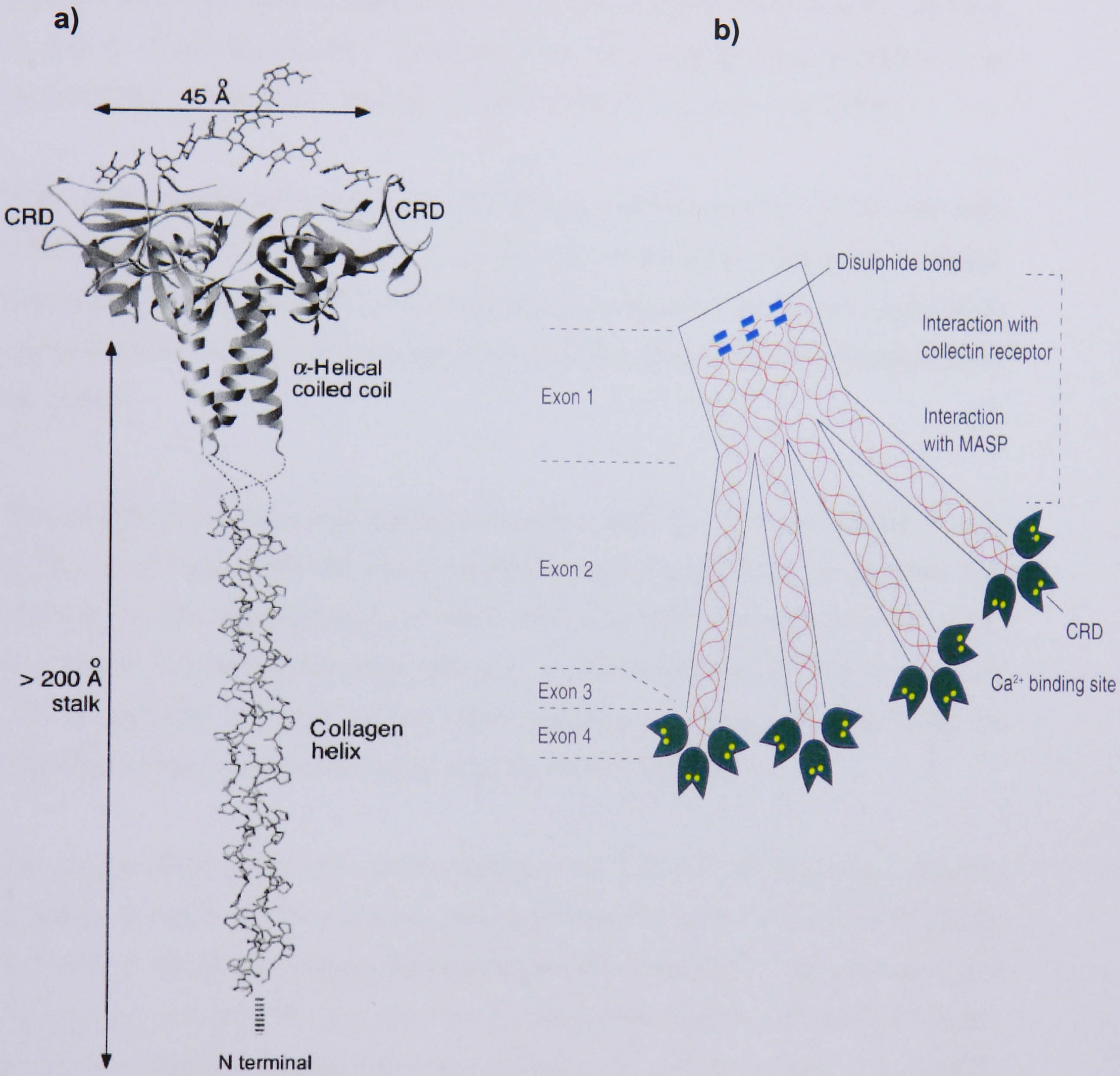


Figure 1.7 MBL Structure. a) Diagram of the human MBL trimer showing the crystallographic CRD domain with the collagen tail. Taken from Ezekowitz (2003). b) Schematic diagram of a tetramer of MBL; note that each of the four subunits is composed of three CRDs. The collagenous regions are known to be involved in interactions with MASP. The regions encoded by the four exons of the human MBL gene are also indicated. Taken from Turner (1996).

In circulation, MBL is able to associate with MASP-1, MASP-2, MASP-3 and a truncated form of MASP-2 known as MAp19 or small MBL associated protein. It

has been shown that the complex MBL-MASP-2 is capable of degrading C4 and C2. This new way to activate the complement cascade is called the lectin pathway. Both MASP-1 and -2 are able to cleave C2, whereas the complex MBL-MASP-1-MAp19 is able to cleave C3 directly. Therefore MBL can, directly through MASP-1 or indirectly through MASP-2, activate C3 (Reviewed by Gadjeva *et al.*, 2001).

It has been reported that when MBL and CRP are working together in the same cell, CRP regulates MBL- initiated cytolysis by modulating alternative pathway recruitment through factor H. This suggests a co-ordinated role for these proteins in complement activation and consequently in the acute phase response (Suankratay *et al.*, 1998).

Interestingly, it has been demonstrated that MBL binds in a calcium dependent way to IgA which also links the innate and the adaptive immunity. The binding was inhibited by the pre-incubation of MBL with mannose or fucose but not in the presence of N-acetylgalactosamine (Roos *et al.*, 2001). Binding of MBL to IgA was able to promote activation of the lectin pathway, a mechanism that might be important in the mucosa where most of the IgA is concentrated.

The human MBL gene lies on chromosome 10. The normal and most common sequence is called allele A, whereas polymorphisms in exon 1 are known as alleles B, C and D. These three single polymorphisms are: at codon 52 (allele D: Arginine replaced by cysteine), 54 (allele B: Glycine replaced by aspartic acid) and 57 (allele C: Glycine replaced by glutamic acid) (Mead *et al.*, 1997; Madsen *et al.*, 1998). These polymorphisms disrupt the assembly of MBL peptide trimers or accelerate the MBL degradation, and result in profoundly reduced serum levels of functional polymeric MBL (Garred *et al.*, 1992b).

Considering only the allele A, the mean MBL concentration in a Danish caucasian population was reported as 1.2 μg/ml (range 0 – 5 μg/ml) (Garred *et al.*, 1992a), but taking into account an allele- mixed population without the allele A levels range from 0 to 1.2 μg/ml in plasma. In heterozygous mutants expressing B or C alleles the serum levels of MBL are reduced to about 1/8 of the wild type level. In contrast,

presence of mutant D reduces MBL to 50% of the wild-type concentration. Subjects homozygous for any of the three mutations usually have undetectable levels of serum MBL (Sumiya and Summerfield, 1996).

Other polymorphisms have been reported in the promoter region of the MBL gene at positions -550 (H/L variants) and -221 (X/Y variants). The LXA haplotype showed decreased levels of MBL comparable to those found in individuals heterozygous for allele B (Madsen *et al.*, 1995). This latter polymorphism adds considerable complexity when MBL concentrations are analysed in a particular population.

Heterozygous and homozygous expression of the different alleles can be found in different populations and are associated with enhanced incidence of infections, in both children and adults (Summerfield *et al.*, 1995; Summerfield *et al.*, 1997). Moreover, homozygous MBL alleles are found in up to 8% in patients suspected to suffer immunodeficiency (Garred *et al.*, 1995).

MBL deficiency is not a well defined term. Genetically altered haplotypes can be present in about 30% of the population. The majority of such individuals are asymptomatic, whereas serum MBL deficiency, which is defined as levels of MBL below 0.1μg/ml, are more rare (Kilpatrick, 2002) and can be the cause of recurrent respiratory infections, such as pneumonia, otitis media, sinusitis in which *Streptococcus pneumoniae* is one of the more frequent etiologic agents (Summerfield *et al.*, 1997).

Symptomatic MBL deficiency is often found in individuals who have another immune defect such as common variable immunodeficiency or IgA deficiency (Mullighan *et al.*, 2000). MBL deficiency also has been associated with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Ip *et al.*, 1998; Graudal *et al.*, 1998).

The first description of a serum defect in opsonisation of *Saccharomyces cerevisiae* in a child with normal neutrophil phagocytic function, but with severe recurrent infections, diarrhoea and failure to thrive, lead to a series of descriptions of children

with similar defects and was associated with low levels of MBL (Super *et al.*, 1989). MBL was shown to opsonise and increase uptake of *Salmonella montevideo* by neutrophils (Super *et al.*, 1992). In the same way MBL-opsonised influenza virus was able to induce production of H_2O_2 in neutrophils (Hartshorn *et al.*, 1993). Neutrophil chemotaxis was affected in patients with MBL deficiency, apparently due to low production of C5a (Ten *et al.*, 1999).

Since MBL is able to bind N-acetyl glucosamine residues, which can be found in the teichoic acid on the C-polysaccharide on *S. pneumoniae*, it is possible that MBL can bind this micro-organism. In patients with recurrent pneumococcal infection, who are suspected to have an immunodeficiency, it is worth considering if the concentration of MBL which might be low or absent. Reconstitution of MBL in patients affected by MBL deficiency might be potentially useful for patients who are suffering from recurrent infections (Valmidarson *et al.*, 1998; Turner , 1998)

1.3.2 C-Reactive Protein

CRP was discovered in 1930, by Tillet and Francis, as a serum precipitin for the C-polysaccharide present in pneumococcal cell walls and capsules, hence the name. CRP is a pentraxin, composed of five identical, non-covalently associated subunits arranged in pentameric radial symmetry (Figure 1.8) (Szalai *et al.*, 1997; Shrive *et al.*, 1996).

CRP is a 110 kDa protein, which contains two faces: first, a calcium-dependent site binding to a widely distributed group of ligands including PCh, polyelectrolytes (Gewurz *et al.*, 1995) and certain phosphorylated polysaccharides (Culley *et al.*, 2000). The main interaction between PCh and CRP occurs between the phosphate group of PCh and calcium molecules of CRP in a shallow pocket of each subunit (Thompson *et al.*, 1999). The other face of the CRP binds to C1q which leads to activation of the classical complement cascade which requires binding to an appropriate multivalent ligand (Volanakis, 1982).

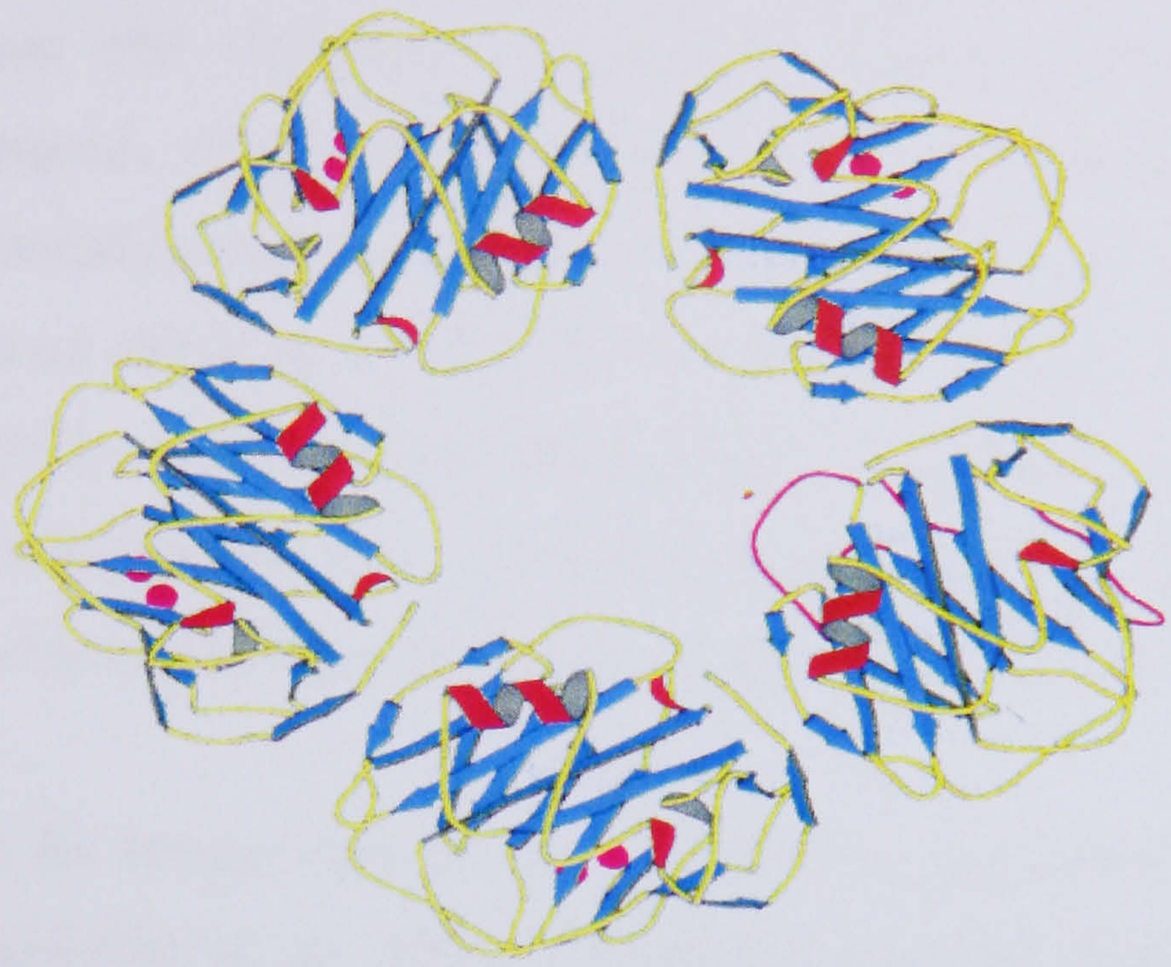


Figure 1.8. Pentameric structure of the human CRP. Pentameric structure of the human CRP viewing the calcium-binding site. Calcium ions are shown in pink. Taken from Shrive *et al.*, (1996).

There are many other reported ligands for CRP in disrupted tissues such as the extracellular matrix proteins fibronectin and laminin via the PCh-binding site (Mortensen and Zhong, 2000). CRP also binds to some ribonucleoproteins, snRNA and the histones H1 and H2 and to a lesser extent to H2B, H3 and H4 (Du Clos, 1989; Robey *et al.*, 1984). Moreover, it has been shown that CRP at 50μg/ml binds to apoptotic cells through nuclear components enhancing their phagocytosis by macrophages. It is suggested that some lysophospholipids, expressed on their membranes may also be involved (Gershov *et al.*, 2000). In this way, CRP participates in the removal of both apoptotic cells and nuclear products from necrotic cells; this effect may have importance in preventing the availability of self-antigens that are involved in the aetiology of autoimmune diseases.

CRP has been reported to protect against various infectious diseases. Rats were protected against *Plasmodium yoelii* infection when the parasites were previously incubated with rat CRP at concentrations from 2.5 to 25 μg/ml (Pied *et al.*, 1989). Pre-treatment of mice with human CRP reduces mortality due to pneumococcal infection serotype 3, an effect that was seen when concentrations reached 9 μg/ml or more (Yother *et al.*, 1982). CRP also protects against *Salmonella enterica* in a transgenic mouse model. Mice carried a 31-kb *Cla*I fragment of human genomic

DNA comprising the CRP gene, 17 kb of 5' flanking sequence, and 11.3 kb of 3' flanking sequence and expressed high levels of CRP in response to injected endotoxin. Transgenic mice experimentally infected with Salmonella lived longer and had lower mortality than non-transgenic mice; after 4 hours post infection CRP concentrations were raised up to 40 μg/ml (Szalai *et al.*, 2000). Many other micro-organisms have PCh on their surfaces and potentially CRP might protect against their infections: e.g. *Clostridium* spp., *Lactobacillus* spp., *Bacillus* spp., *Haemophilus influenza*, *Neisseria meningitidis* and *Pneumocystis carinii*. (Szalai, 2002a).

The single gene for human CRP has been located in chromosome 1 in the region 1q21-q23 (Floyd-Smith *et al.*, 1986). Nucleotide sequence analysis indicates that after coding for a signal peptide of 18 amino acids and the first two residues of the mature protein, there is an intron of 278 base pairs followed by the nucleotide sequence for the remaining 204 amino acids (Woo *et al.*, 1985).

Most of the increase in CRP plasma levels during the acute-phase response results from transcriptional control in the liver. The transcription of CRP is controlled by two CCAAT enhancer binding protein (C/EBP) sites, two glucocorticoid-responsive elements, an IL-6-responsive element (IL-6RE), two hepatocyte nuclear factor –1 elements (Gewurz *et al.*, 1995; Toniatti *et al.*, 1990) and Stat 3 (part of the signalling induced by IL-6). For example in a mouse model, inactivation of Stat 3 in the liver led to significant impairment of the acute phase response (Alonzi *et al.*, 2001).

Cytokines such as IL-6, IL-1 and TNFα, which are known to increase the concentration of CRP in plasma, act through intracellular pathways that ultimately promote binding of transcriptional factors to their respective control elements in the DNA to generate mRNA and the protein itself (Cha-Molstad *et al.*, 2000).

There is one report on polymorphisms in the CRP gene although it does not have any effect at the amino acid level of the protein (Cao and Hegele, 2000). A polymorphic $(GT)^{9 \text{ to } 25}$ repeat found in the intron (Woo *et al.*, 1985) which correlates with baseline levels of CRP and with differences between concentrations found in

Caucasians and Afroamericans was also reported (Szalai *et al.*, 2002b). However, there also might be an enhancer variation of either tissue specific or inducer regions.

1.3.2.1 CRP and neutrophil movement *in vivo*

In experimental and disease-caused lesions, the accumulated CRP is associated specifically with neutrophils (Du Clos *et al.*, 1981; Kushner and Kaplan, 1961). However, there are many controversial reports about the effects of CRP on neutrophils, some of the effects are proinflammatory and include the induction of respiratory burst (Potempa *et al.*, 1988; Zeller and Sullivan, 1992). However, the net reported effect, on neutrophils at least, is anti-inflammatory.

In mice transgenic for rabbit CRP with serum levels ranging from 75 to 200μg/ml, resistance to intraperitoneal LPS or intravenous PAF was greater than in wild type mice. This effect was partially explained by the ability of CRP to bind to PAF (Xia and Samols, 1997), but also, may be explained by its ability to stimulate the synthesis of IL-1RA by mononuclear cells (Tilg *et al.*, 1993). Moreover, in a model of Experimental Allergic Encephalomyelitis in transgenic mice expressing human CRP, CRP at high concentrations is able to decrease the production of MIP1α, RANTES, MCP-1, TNFα and IFNγ and increase IL-10. This effect is correlated with delay in the clinical course of the disease and little infiltration in the spinal cord, especially in male transgenics (Szalai *et al.*, 2002c).

CRP at the lower end of acute phase concentrations (between 10 and 50 μg/ml) were reported to inhibit neutrophil chemotaxis induced by fMLP and IL-8 (Zhong *et al.*, 1999) whereas another report suggested that concentrations between 50-200 μg/ml are required for inhibition of fMLP induced migration (Heuertz *et al.*, 1999). However both reports show effects only at acute phase concentrations. These reports also show CRP dependent changes in signalling events which may lead to the responses seen. Acute phase levels of CRP inhibited p38 MAPK activation which is important for chemotaxis (Heuertz *et al.*, 1999). The significance of the ability of CRP to induce ERK and PI_3K in relation to chemotaxis is unclear (Zhong *et al.*, 1998).

1.3.2.2 CRP and its receptors

CRP binds to different receptors on neutrophils, FcγRI, and maybe also to FcγRIIA which is found on resting cells (Kilpatrick and Volanakis, 1985; Crowell *et al.*, 1991; Bharadwaj *et al.*, 1999). It has been reported that a polymorphism of FcγRIIA may affect CRP-binding to neutrophils, an arginine (RR) at position 131 facilitates CRP binding but decreases IgG2 binding. The opposite effect occurred when a histidine (HH) was present at that particular position, (Stein *et al.*, 2000b) although this result is controversial.

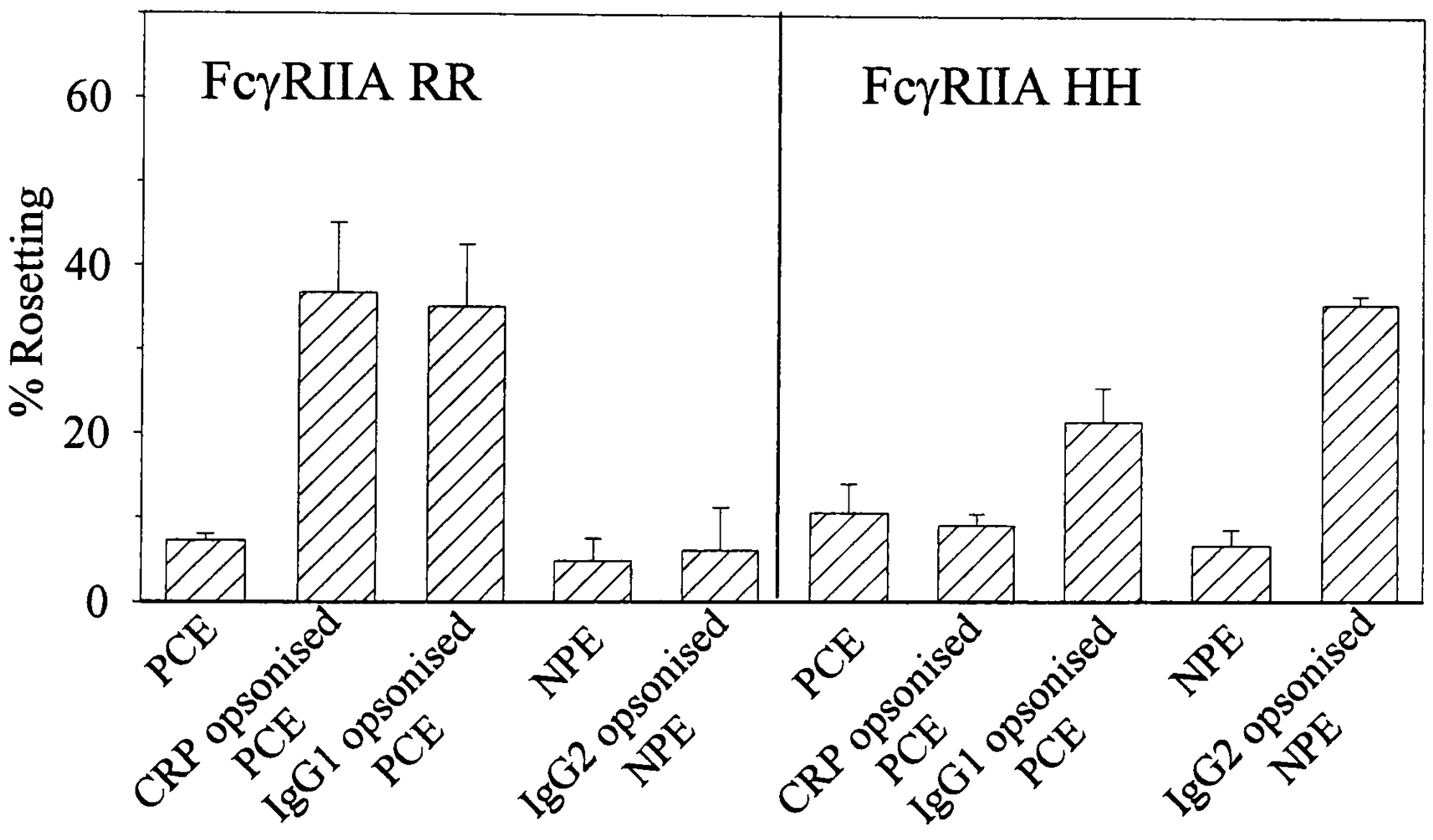


Figure 1.9 CRP-opsonised SRBC binds FcγRIIA –RR but not –HH transfected Cos cells. PCh coupled erythrocytes were opsonised with IgG1 (against stroma of SRBC) or CRP and incubated with FcγRIIA RR or HH transfected Cos cells. As a positive control for FcγRIIA-HH binding, erythrocytes were coupled to NP and opsonised with IgG2 anti-NP. After incubation, cells were washed, fixed and stained for erythrocytes. Bodman Smith K,Gregory R. E, Rodriguez J. A and Raynes J. G. (unpublished observations)

CRP binding to FcγRIIA is controversial, since the technique used to analyse binding was dependent on using whole detecting antibodies which can also bind to FcγRI or RIIA H or R through the Fc portion creating a false positive result. In fact, the use of

a $F(ab')_2$ portion of the same antibody could not reproduce the results (Saeland *et al.*, 2001). However, if single monomeric (pentamer) CRP bound with low affinity to RIIB one would not see binding using this technique since the off-rate of binding would be very high in a monomeric interaction.

CRP binding to FcγRIIA was confirmed by Dr K Bodman-Smith who analysed binding of CRP-opsonised PCh-labelled SRBC to FcγRIIA transfected-Cos cells (Figure 1.9). When FcγRI was co-transfected with FcγRIIA there was an increase in phagocytosis, but when FcγRIIB was present phagocytosis was inhibited (Bodman Smith K., Gregory R. A., Rodriguez J. A. and Raynes J. G., unpublished observations).

It is well known that IgG2 is very important in the control of some encapsulated bacteria, such as *Streptococcus pneumoniae,* indeed children who have IgG2 deficiency are prone to pneumococcal infections (Barrett and Ayoub, 1986). From this, it is assumed that the phagocytes of children homozygous for H131 are better able to bind IgG2 and consequently have better control of pneumococcal infections than children with R131 alleles. RR children are probably more dependent on CRP binding to *S. pneumoniae*, and any variation of CRP may increase the frequency of infections in those already predisposed.

Since atherosclerosis has an inflammatory component, CRP has been implicated as a risk factor especially at moderately raised levels. However, whether CRP is involved in the pathogenesis of the disease remains unclear. It is possible that its elevation may be a consequence rather than a cause of the process (Williams and Tabas, 2002).

1.3.3 Serum amyloid P

Another member of the pentraxin family is serum amyloid P (SAP). CRP and SAP are distinguished by their calcium dependent ligand specificities; CRP binds to PCh whereas SAP binds to phosphorylethanolamine. SAP also binds to a range of carbohydrates (Loveless *et al.*, 1992). Human CRP and SAP share approximately

66% amino acid homology and there are major differences in their tertiary structure as was described by Shrive *et al.*, (1996).

SAP in humans is constitutive and therefore does not increase in concentration or only increases slightly during an acute phase response (human serum concentration is about 40 μg/ml). However, in mice SAP behaves as an APP while CRP is constitutive and is present at low concentrations. SAP also activates the classical complement pathway.

SAP binds to heparin, laminin, fibronectin, C4 binding protein; human SAP has been shown to bind to murine FcγRs, and was reported to bind to all three receptors, mFcγRI, mFcγRII and mFcγRIII whereas human CRP bound to mFcγRI and mFcγRII (Mold *et al.*, 2001).

SAP has been shown to bind to nuclear components such as chromatin and histones, and recently was shown to opsonise apoptotic cells to help their phagocytosis by macrophages (Bijl *et al.*, 2003). Human SAP and CRP at 100 μg/ml have also been shown to help in the clearance of apoptotic Jurkat cells and the receptors involved in clearance were FcγRI and FcγRIII for CRP and SAP respectively (Mold *et al.*, 2002).

Although knockout SAP -/- mice replete with human SAP had better survival when compared with those depleted when exposed to lethal doses of *E. coli* O111:B4 (a strain to which human SAP does not bind) SAP -/- mice survived longer than the wild type mice following lethal doses of *E.coli* and *Streptococcus pyogenes* (both of which bound to human SAP). In the same study human SAP at increasing concentrations from 0.1 to 1000 μg/ml decreased phagocytosis of *Neisseria meningitidis* by human neutrophils (Noursadeghi *et al.*, 2000).

These apparently contradictory results might arise from the use of a human protein in a mouse model where it does not have the same affinity for FcγRs. Research on the interaction between CRP and FcγRs will help in understanding early events in inflammation.

1.4 Aims

Since neutrophils and APPs such as MBL and CRP are present together during the process of the inflammatory response, it is very likely that they interact on many occasions in order to alter the immune response. Although is also possible that interactions take place when there are normal serum concentrations of APPs, it has been reported that different concentrations may have different effects on neutrophil physiology. CR1 has been identified as a receptor for MBL and FcγRs have been reported as receptors for CRP. However it is still unclear what might be the role of FcγRII and polymorphisms of FcγRIIA in the interaction with CRP. *Streptococcus pneumoniae* has been chosen as a model to study some of these issues because of its capacity to bind to CRP and because neutrophil defects are associated with recurrent pneumococcal infections such as in neutropenic patients, hyper-IgE syndrome and Chediak-Higashi syndrome among others. Therefore the main aims of this thesis are:

1. To investigate the capacity of CRP and MBL to alter neutrophil functions when they are in contact with these cells, either alone or in the presence of *Streptococcus pneumoniae.*
2. To analyse if there are variations in the response by neutrophils when normal serum concentrations or acute-phase concentrations of CRP and MBL are used, either alone or in the presence of *Streptococcus pneumoniae.*
3. To clarify the preference of CRP in terms of binding to FcγRIIA on neutrophils from individuals expressing the HH or the RR polymorphism. The logical extension of the report by Stein et al., 2000b being that individuals expressing H allele alone will fail to respond to CRP. A panel of normal donors were typed for the polymorphism and tested in a variety of assays including NADPH oxidase activity, cytokine production and phagocytosis.
4. To test whether CRP at different concentrations is able to bind to FcγRIIB or other Fcγ receptors.
5. Compare recombinant and purified CRP in aims 1, 2, 3 and 4 because of reports of effects due to contaminating IgG.

2. MATERIALS AND METHODS

2.1 Isolation of Mannose Binding Lectin and C-Reactive Protein

2.1.1 Purification of MBL

Purification of MBL was achieved using methodology similar to that described by Colley *et al.*, (1988) with minor modifications.

Two litres of plasma from plasmapheresis-patients were obtained from University College Hospital. Plasma was converted to serum by antagonising the heparin anticoagulant effect with 40ml/L of a mixture of 1.66 mg/ml protamine sulphate (Sigma-Aldrich, Dorset, UK) and 0.33M $CaCl_2$. Plasma was left to stand at 37°C for 1 hour in a centrifuge bucket to coagulate, and then transferred to 4°C for a further 2 hours. The mixture was centrifuged at 3500g (J-6B Centrifuge, Beckman, USA) for 2 hours, then the supernatant was transferred to a clean container and 10mM $CaCl_2$ was added and incubated overnight at RT.

On the following day, precipitation was observed, and the serum was re-centrifuged at 3500g (J-6B Centrifuge, Beckman, USA) for 5 minutes and the supernatant was transferred to a different container. At the same time, 10 ml of mannan-coated cross-linked 4% beaded agarose (Sigma-Aldrich, Dorset, UK) were washed with 8 mM Tris HCl pH 7.5 containing 0.15M NaCl (TBS) containing 20mM $CaCl_2$. The mannan-agarose beads were transferred to the serum and left at RT for 3 hours with shaking from time to time.

Mannan-covered beads were recovered again by passing serum through a scintered glass funnel (G3), transferred to a column and then washed with TBS/20mM$CaCl_2$. Then protein attached to mannan-covered beads in a calcium dependent manner, was eluted with TBS containing 5mM EDTA; 3 ml-fractions were collected and OD_{280} determined in a spectrophotometer (Perkin-Elmer, UK).

Those fractions that contained protein were collected and dialysed against TBS for 1 hour and then overnight. The column was washed first with TBS containing 20mM $CaCl_2$ and then with TBS containing 10 mM $CaCl_2$. The dialysis product was then incubated with the beads in the column for 2 hours at RT. After that, 10mM TBS/$CaCl_2$ + 50mM Mannose were added and fractions were collected and OD_{280} determined. Finally, those fractions that contained protein were concentrated by centrifugation in Centricon® tubes to approximately 0.5 μg/ml (Molecular weight cut off -10000 daltons Amicon, Watford, UK). Typically 70 μg of MBL were purified from 2000 ml of plasma, and the protein concentration determined by ELISA.

2.1.2 Assessment of protein purity: SDS polyacrylamide gel electrophoresis and western blotting

A vertical gel electrophoresis system was used (Mini PROTEAN III dual slab cell, Bio Rad Laboratories, Ca, USA). The gel apparatus was set up according to the manufacturer's instructions.

12% (w/v) SDS-PAGE was performed as follows: A denaturing gel was composed of 1)resolving gel: 3 ml of 40% (w/v) acrylamide (1:19 v/v ratio of bisacrylamide to acrylamide) (Severn Biotech Ltd, Kidderminster, UK), 2.5 ml of 1.5M Tris-HCl (pH 8.8), 0.13 ml of 10% (w/v) sodium dodecyl sulphate –SDS (BDH, Poole, Dorset, UK) and 200 μl of 1.5% of fresh ammonium persulphate $(NH_4)_2SO_4$ (Sigma – Aldrich, Gillingham, UK) and then 10 μl of NNN'N'-tetra-methylethylendiamine (TEMED)(Sigma-Aldrich, Gillingham, UK) to initiate polymerisation. Immediately after the addition to the casting apparatus, the gel was overlaid with distilled H_2O. After setting, the gel was washed, the interface dried and stacking gel was added ; 2) the stacking gel was composed of: 1ml of 40% acrylamide, 2.5 ml of 0.5M Tris-HCl (pH 6.8), 100 μl of 10% (w/v) SDS and 6ml of H_2O, plus 50 μl of 1.5% (w/v) of fresh ammonium persulphate and finally 10 μl of TEMED. A comb was immediately added and when set, the wells were washed with dH_20.

Pre-stained markers of known molecular weight were used (Sigma - Aldrich, Gillingham, UK) consisting of: β-galactosidase, *E. coli* 116kDa; bovine serum albumin 66kDa; chicken egg ovalbumin 45kDa; carbonic anhydrase 29kDa, soy bean trypsin inhibitor 20.1kDa; α-lactalbumin 14kDa and aprotinin 7.5kDa. Each vial of color markers contained 500 μl of a mixture of the protein-dye conjugates in 62 mM Tris-HCl, pH 7.5, containing 2% (w/v) SDS, 0.1 mM EDTA, 100 mM dithiothreitol, 4M Urea, 0.005% (w/v) bromophenol blue and 30% (v/v) glycerol.

10 μl of each sample were mixed with 15 μl of sample buffer (62 mM Tris-HCl, pH 7.5, containing 2% (w/v) SDS, 0.1 mM EDTA, 100 mM dithiothreitol, 0.005% (w/v) bromophenol blue and 30% (v/v) glycerol) and boiled for two minutes. Immediately 20 μl of the mixture were loaded into the well. Electrophoresis was carried out under constant voltage of 100V for approximately 1 hour, using 25 mM Tris base containing 192 mM Glycine and 0.1% (w/v) SDS as the running buffer.

Gels were either stained with Coomassie Blue which was carried out with 0.125% Coomassie blue R250 (w/v) in 50%(v/v) methanol, 10% (v/v) acetic acid 40% (v/v) H_2O or protein was transferred to a polyvinyldifluoride (PVDF) membrane using a semi dry blotting apparatus (Sartoblot IL-S, Sartorius, GB-Belmont, Surrey) with recommended buffer: 20mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol. The apparatus was run at 10V for 1 hour. A biotinylated mouse monoclonal antibody against MBL at 1/3000 dilution in TBST (20mM Tris-HCl, pH 8.0, 137 mM NaCl containing 0.1% (v/v) Tween 20 for 2 hours) (Statens Serum Institute, Copenhagen, Denmark) was added to the PVDF following the blocking stage with 5% (w/v) skimmed milk in TBST. The membrane was washed and developed using streptavidin:alkaline phosphatase (1/3000) (Serotec, Oxford, UK) and nitro blue tetrazolium with 5-bromo-4-chloro-3- indolyl phosphate (NBT/BCIP-Sigma-Aldrich, Gillingham, UK) as substrate diluted in 0.1M Sodium Hydrogen Carbonate buffer pH 9.6 containing 2mM $MgCl_2$.

2.1.3 Determination of the concentration of MBL

An ELISA procedure was used to determine the concentration of MBL in serum samples. The technique was similar to that described by Thiel *et al.*, (1992). 96-well flat bottom plates (Immulon 2HB, Dynex technologies, UK) were coated overnight at 4°C, with 100 µl of a rabbit polyclonal antibody anti- human MBL, (provided by Dr Jacques U Baenziger, Washington University Medical School), diluted 1/5000 in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH 9.6).

After 3 washes with TBS containing Tween 0.05% (v/v)(TBST), plates were blocked by adding TBST 10% (v/v) FCS for 30 minutes at room temperature. After 3 washes with TBST, 50 µl of samples (previously diluted 1/100 in TBST containing 5 mM EDTA) and standards were added and left overnight at 4°C. A standard curve was made with MBL (covering a range 70 ng/ml to 1.09 ng/ml) provided by Professor MW Turner at The Institute of Child Health, London, UK. After 3 more washes with TBST, 50 µl of a mouse biotinylated monoclonal antibody to human MBL (1/5000) (Statens Serum Institute, Denmark) were added and the plates left for 2 hours at room temperature (RT).

Following a further 4 washes, 50 µl of horseradish peroxidase (HRP)-streptavidin (Dako SA, Denmark) were added for 1 hour at RT. Plates were washed 3 times with TBST and 3 more times with TBS alone and then 50 µl of substrate, 0.1 mg/ml Tetramethylbenzidine- (TMB, Sigma-Aldrich, Gillingham, UK) in citrate buffer pH 4.5 (0.1 M Citric acid and 0.1 M Na_2PO_4 diluted 2 volumes of acid plus 3 volumes of base and then activated with 2µl H_2O_2) were added for 30 minutes. The reaction was stopped with 2M sulphuric acid and plates read at 450nm on an ELISA reader with a control wavelength at 490 nm (MRX Microplate Reader, Dynex technologies, UK).

2.1.3.1 Cohort of Colombian children

Children were chosen from the recurrent infection clinic at the Hospital Universitario "Hernando Moncaleano Perdomo" in Neiva, Huila, Colombia over the period from December 1996 to December 1999. A total of 11 children were selected because of

recurrent pneumococcal respiratory infections and were evaluated for pneumococcal antibodies against the different serotypes. None of these patients were younger than one year of age and all of them were between 2 to 5 years of age. This cohort of patients was studied after obtaining informed consent from their parents. The procedure followed the guidelines provided by the ethical committee of the Hospital and Universidad Surcolombiana.

2.1.3.2 Immunological studies

Serum samples were collected after obtaining informed consent. To study total serum concentration of immunoglobulins IgM, IgA, IgG, IgG subclasses and C3, C4, samples were analysed by nephelometry using reagents provided by Boehringer NF100 (Merck, USA). Samples were stored at -20°C until values were determined.

2.1.3.3 Pneumococcal antibodies

A group of 11 children with recurrent pneumococcal infections were vaccinated with 23-valent polysaccharide pneumococcal vaccine Pnu-Immune 23 (Lederle, Pearl River, NY which contains 25μg of each of the following capsular polysaccharide (pcps) types: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) according to published criteria (Sorensen *et al.*, 1998) (Table 2.1). 10 ml of blood were drawn before and 4 –weeks after vaccination.

Table 2.1 Indications for the use of the pneumococcal vaccine. From Sorensen *et al.*, (1998).

Disease	**# episodes/year (children between 2 to 5 years)**	**# episodes/year (children older than 5 years)**
URI* with antibiotics	>4	>2
Otitis with antibiotics	>3	>2
Sinusitis	>2	>1
Chronic, resistant sinusitis	>1	>1
Pneumonia	>2	>2
Need of preventative antibiotics	≥1	≥1
Allergy to antibiotics for treatment of respiratory infections	≥1	≥1

* Upper respiratory tract infections

IgG specific for different pneumococcal capsular polysaccharides were determined by ELISA following the method described by Koskela and Leinonen (1981) with some modifications stated by CDC to unify technical procedures (Quataert *et al.*, 2001). Briefly, ELISA plates (Nunc Maxixorb, USA) were coated with 100 µl of different pcps: 1, 4, 5, 6B, 9V, 14, 18F, 19 and 23F (ATCC, Rockville, MD, USA) diluted in 0.01 M Na_2HPO_4/KH_2PO_4 pH 7.2, for 5 hours at 37ºC and then stored at 4ºC until used.

The following day plates were washed three times with PBS-Tween. Standard serum FDA 89-SF was kindly provided by Carl R. Frash (CBER; US Food and Drug administration, Rockville, Md). The concentration of IgG specific for pneumococcal polysaccharides in this serum was already established (Quataert *et al.*, 1995). Serum samples and reference serum were incubated with C-polysaccharide (Statens Serum Institute, Denmark) to remove contaminating antibodies (Musher *et al.*, 1990) before adding them to the plates. Samples from children were diluted 1/50 and serial dilutions were performed up to 1/400, whilst 89-SF serum was diluted 1/200 and serial dilutions were performed up to 1/12800 for IgG determinations. 50 µl of each dilution of standard and samples were added to the plates and were incubated for 30 minutes at 37ºC. After three washes with PBS-Tween an HRP-conjugated mouse IgG (diluted 1/1000) to human IgG (ICN Biochemical, USA) was added for 1 hour at RT. Finally, 50 µl of tetramethylbenzidine (Sigma-Aldrich, USA) were used as a substrate diluted in citrate buffer pH 4.5 (0.1M citric acid and 0.1M Na_2PO_4 diluted in 2 volumes of acid plus 3 volumes of base) plus 2 ml of H_2O_2 to activate the substrate immediately before use. The reaction was stopped with H_2SO_4 and read at 450 nm using a 690 nm reference filter, with an ELISA reader (ELX 800, Bioteck, USA).

2.1.4 Isolation of C-reactive protein

CRP (pCRP) was extracted from human acute phase serum using a protocol described by Culley *et al.*, (1996).

1)Affinity chromatography: Phosphorylcholine Sepharose was prepared by coupling ρ-aminophenylphosphorylcholine (Sigma-Aldrich, Gillingham, UK) dissolved to activated CH-Sepharose 4B (Pharmacia, Milton Keynes, UK). 4g of activated CH-Sepharose 4B were swollen in 20 ml of 1mM HCl for 15 minutes at RT. Sepharose was then washed four times at 300-500 rpm at 10°C with ice-cold 1mM HCl (approximately 500 to 1000 ml). Sepharose was then incubated with ρ-aminophenylphosphorylcholine in 20 ml of coupling buffer (0.1M NaHCO3, pH 8.0 containing 0.5M NaCl) for 1 hour at RT. The gel was washed four times and then excess reactive groups reacted with Tris-HCl (0.1M, pH8.0) for 1 hour standing at RT, inverting it occasionally. The gel was washed with 3 cycles of 100ml of sodium acetate (0.1M, pH4.0 containing 0.5M NaCl) then Tris-HCl (0.1M, pH8.0 containing 0.5M NaCl). Sepharose was equilibrated in TBS and stored at 4°C in TBS containing 0.01% NaN3.

60 ml of phosphorylcholine-sepharose suspension were washed with 200 ml of TBS (pH 8.0), containing 1 mM $CaCl_2$ (TBSC), and added to approximately 500 ml of acute phase serum (a sample of which was retained), mixed on a rotator at 4°C for 16 hours, after which the mixture was poured onto scintered glass over a vacuum. The beads were retained, washed with 1000 ml of TBSC, packed into a column and washing continued until the OD_{280} of the eluted buffer fell below 0.01. CRP was eluted with TBS containing 10mM ethylenediaminetetra-acetic acid salt (EDTA) pH 8.0, and the major protein containing fractions were pooled.

2)Ion exchange chromatography: 100 ml of 2:1 v/v TBS:dH_2O was run through a 10g column of Whatman pre-swollen microgranular anion exchanger (diethylaminoethyl cellulose-52), followed by the CRP samples obtained above which had been previously diluted to 0.1M NaCl and the column washed with 10mM Tris pH 8.0 0.1M NaCl. CRP was eluted with a linear gradient from 0.1M to 1M NaCl, 10 mM Tris-HCl, pH8.0, collected in 5 ml fractions, the OD_{280} determined and the peak fractions pooled.

3)Gel filtration: Fractions were obtained and dialysed overnight against phosphate buffered saline, 140mM NaCl, 8mM Na_2PO_4, 1.5mM KH_2PO_4, 2.7 mM

KCl, pH 7.4 (PBS) and concentrated to a volume of approx 5 ml using polyethylene glycol 20,000 (BDH) and run through a 300 ml Sephacryl-300 column (Pharmacia), followed by degassed PBS, at a flow rate of 2ml/min and collected in 6 ml fractions. The OD_{280} was determined and the peak fractions were pooled. The void volume (V_0) and the total volume (V_t) of the column were determined using blue dextran (MW= $2x10^6$) (Sigma-Aldrich, Gillingham, UK). Bovine serum albumin fraction V (BSA)(Sigma-Aldrich, Gillingham, UK) was also run as a molecular weight marker.

The concentration of CRP in the final sample was obtained by measuring the OD_{280}; CRP (mg/ml)= $OD_{280\ 1cm}$ / 1.98 (Gotschlich and Edelman, 1965). The purity was assessed by 10% SDS-PAGE similar to the procedure described in section 2.1.2 using the following molecular markers: Rabbit muscle myosin, 205 kDa; β-Galactosidase, *E. coli* 116kDa; Bovine serum albumin, 66 kDa; Chicken egg ovalbumin, 45kDa; Bovine erythocyte carbonic anhydrase, 29 kDa; Soybean trypsin inhibitor, 20.1kDa.

Human recombinant human CRP (rCRP), expressed using a plasmid in *Escherichia coli* and purified by affinity chromatography, (Tanaka *et al.*, 2002), kindly provided by Dr Toshio Tanaka (Nagahama Institute for Biochemical Science, Oriental Yeast Co., Ltd., 50 Kano-Cho, Nagahama-shi, Shiga 526-0804, Japan) was used in some experiments to compare responses with pCRP. The preparation was reported as containing 9 ng of LPS per 1 mg of rCRP (Personal communication from Yuhsi Matuo).

2.1.5 Isolation of SAP

SAP was provided by Dr J. Raynes. The protein was isolated on phosphoryl ethanolamine- Sepharose 4B and eluted with TBS 10 mM EDTA. Anion exchange chromatography (DE52) and gel filtration (S-300) were then used to further purify the protein according to methods described previously (Loveless *et al.*, 1992).

2.2 Isolation of Peripheral Blood Mononuclear Cells and Neutrophils

2.2.1 Donors

Healthy donors, older than 18 years of age from the London School of Hygiene and Tropical Medicine, who volunteered to give blood for the study, were recruited for the different experiments performed in this study. An informed consent was signed by each of them and the procedure was approved by the Ethical Committee of the School.

2.2.2 Histopaque Purification: Blood from healthy volunteer donors was collected in tubes containing in 10 IU /ml heparin, diluted 1/3 with RPMI 1640 (Gibco BRL-Life technologies, Paisley, UK) and carefully layered onto a Histopaque density gradient (15 ml of Histopaque-1.116 at the bottom and 15 ml of Histopaque-1.070 at the top)(Sigma-Aldrich, Gillingham, UK) of a 50ml-falcon tube. Tubes were centrifuged at 700 g for 30 minutes; this procedure allows separation of peripheral blood mononuclear cells (PBMCs) from polymorphonuclear cells (PMN), since the latter remain at the interface between the two different densities of histopaque (Ferrante and Thong, 1978). Both layers were recovered and washed with RPMI 1640.

PBMCs were recovered washed three times with RPMI and stored at 4°C to use as a source of DNA purification. Treatment with 5 ml of distilled water for 30 seconds and then rapid restoration of isotonic conditions with RPMI lysed contaminant red blood cells in PMN layer. Two more washes were performed at 200 g. Viability of the cells was always more than 95% according to trypan blue staining. Cells were counted in a Hemocytometer and adjusted to 2×10^6/ml.

2.2.3 Dextran Purification: 30 ml of undiluted blood were added to a falcon tube containing 5 ml-Acid Citrate Dextrose solution (ACD-Sigma-Aldrich, Gillingham, UK). Blood was mixed carefully and transferred to a tube containing dextran solution: 3% dextran (Dextran T 500 Pharmacia, Milton Keynes UK) + 0.9% (w/v) NaCl in a ratio 1:2 (dextran: blood). The mix was allowed to sediment for 30 minutes at room temperature and the supernatant was centrifuged at 300g for 5 minutes and

resuspended in 15 ml of cold CD-Hybridoma medium (Gibco, Paisley, UK). Then it was carefully layered on 15 ml of Histopaque – 1.070 and centrifuged for 30 minutes at 400g at 4°C to remove PBMCs.

Two washes with distilled water for 30 seconds as described before were used to eliminate contaminant red blood cells. Neutrophils were counted and resuspended in CD- hybridoma medium at the concentration required, usually between 1 to 6 per 10^6 cells / ml. More that 95% were confirmed as polymorphonuclear cells by staining with Wright–Giemsa for 5 minutes at RT (Sigma-Aldrich, Gillingham, UK). Viability was more that 97%.

2.3 Apoptosis Assays

2.3.1 Annexin V

Neutrophils were incubated in CD hybridoma medium for up to 24 hours with or without 100 IU/ml of IFNγ. In a different experiment neutrophils were incubated overnight at 37°C with *Streptococcus pneumoniae* at a ratio 30:1 in the presence of CRP at different concentrations: 10, 50 and 100 μg/ml. See section 2.5.1 for more details about culture of pneumococci. In all these experiments Annexin V binding was determined as follows:

Neutrophils were resuspended at 1 x 10^6/ml in cold PBS, and then 100 μl were transferred to a 5 ml falcon tube. 5 μl of Annexin V-FITC (BD Biosciences, USA) were added at the same time as 10 μl of propidium iodide (BD Biosciences, USA). Cells were gently vortexed and incubated at RT in the dark for 20 minutes. 400 μl of buffer containing 10 mM Hepes (pH 7.4), 140 mM NaCl, 2.5mM $CaCl_2$ were added to each tube; and at least 20000 events were immediately acquired by flow cytometry (Facs Scalibur, BD Biosciences, USA) within 1 hour.

2.3.2 Terminal UTP nick end labelling (TUNEL)

Isolated neutrophils at 1 x 10^6 per condition were incubated in 5 ml falcon tubes containing CD hybridoma medium and incubated at 37°C for 1, 4 , 18 and 24 hours in the presence or absence of 100IU of IFNγ. The following protocol was used according to the manufacturer's guidelines (R&D Systems, Abingdon, Oxon, UK):

1 x 10^6 cells were centrifuged at 500g for 5 minutes at room temperature (RT) in 5 ml falcon tubes. The cell pellet was gently resuspended in 1 ml of 3.7% (w/v) formaldehyde (Sigma-Aldrich, Gillingham, UK) and incubated for 10 minutes at RT, then centrifuged at 500g for minutes. The pellet was resuspended in 1 ml PBS and left at RT for 5 minutes, before centrifugation at 500g for 5 minutes. To the new pellet 100µl of permeabilisation and blocking agent provided (Cytonin^{TM}) were added and incubated overnight at 4°C. After centrifugation for 5 minutes at 500g, 1ml labelling buffer (containing 100mM terminal deoxynucleotidyl transferase (TdT) Buffer, 0.5mg/ml bovine serum albumin and 0.6 mM 2- mercaptoethanesuphonic acid) was added to each tube and centrifuged again for 5 minutes at 500g. 25µl of labelling reaction mixture (1 µl of TdT containing 25 units, 1 µl of a mixture of biotinylated dNTPs and 1 µl $MnCl_2$ in 50 µl of buffer 0.1% (v/v) Triton X-100 and 5 mg/ml BSA in PBS) were added to each tube and incubated for 1 hour at 37°C.

1 ml of 10mM EDTA pH 8.0 was added to stop the reaction and each tube was centrifuged at 500g for 5 minutes at RT. Finally, 25 µl of streptavidin-fluorescein isothiocyanate (FITC) were added and left for 10 minutes in the dark at RT. Cells were washed twice with PBS at RT and resuspended in 500 ml of PBS and left on ice until acquired by FACS (FACS SCALIBUR, Becton Dickinson, San Diego, USA).

As a positive control formaldehyde fixed-neutrophils were treated with 25 µl of endonuclease for 30 minutes at 37°C and then washed with 1 ml labelling buffer for 5 minutes at 1500g at RT. Then 25 µl of labelling reaction mixture were added as described above. As a negative control TdT was omitted from the labelling reaction mixture.

2.4 Polymorphisms of FcγRIIA

2.4.1 Purification of DNA

DNA was extracted according to the procedure included in the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). 300 µl of blood sample were added to 1.5ml-eppendorf tubes, 900 µl of cell lysis solution were added to lyse red blood cells and the tubes inverted 5-6 times to mix. After 10 minutes incubation at RT, tubes were centrifuged at 13000g for 20 seconds. Supernatant was discarded and pellets containing white blood cells were vortexed for 10 -15 seconds and resuspended in 300 µl of nuclei lysis solution and pipetted 5-6 times until it was very viscous. Then, proteins present in the solution were precipitated by adding 100 µl of protein precipitation solution, vortexed for 20 seconds until small protein clumps were observed.

After centrifugation for 3 minutes at 13000g, a dark brown protein pellet was observed, and the supernatant was transferred to a 1.5 ml- eppendorf tube containing 300 µl of isopropanol. The solution was mixed by inversion until DNA formed a visible mass and the tube centrifuged again for 1 minute at 13000g. To the pellet, 300 µl of 70% ethanol were added and centrifugated for 1 minute at 13000g. Finally, to rehydrate DNA, 100µl of a solution containing 10mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8.0) were added and left overnight at 4°C.

The purity of the product was verified by electrophoresis in 0.85% agarose (Bioline, London, UK). DNA was stored at 4°C before analysis.

2.4.2 PCR to determine FcγRIIA polymorphisms

PCR was performed to amplify specific FcγRIIA sequences of DNA based on a method described elsewhere (Flesch *et al.*, 1998). A PCR mix was prepared containing 200µM of each dNTP (Bioline, London, UK), 0.5 U of Taq polymerase (Bioline, London, UK), 0.125 µM of human growth hormone gene as an internal control HGH - HGH2 CAGTGCCTTCCCAACCATTCCCTTA and ATCCACTCACGGATTTCTGTTGTGTTTC (Sigma-Genosys Ltd, Cambridgeshire,UK) 0.5 µM common antisense primer from an intron shared by the

sequences for FcγRIIA, FcγRIIB and FcγRIIC CAATTTTGCTGCTATGGGC (Sigma-Genosys Ltd, Cambridgeshire, UK), and 0.5 μM of H131-specific sense primer ATCCCAGAAATTCTCCCA (Sigma-Genosys Ltd, Cambridgeshire,UK) (Hmix) or R131-specific sense primer ATCCCAGAAATTCTCCCG (Sigma-Genosys Ltd, Cambridgeshire,UK) (Rmix) in 10X buffer and 15mM $MgCl_2$ (Bioline, London, UK)

22.5 μl of either Hmix or Rmix were used in combination with 2.5 μl of DNA sample. PCR was performed on a thermal cycler (Omnigen, Hybaid LTD, UK) as follows: 10 minutes at 95°C, 10 cycles of 1 minute at 96°C, 2 minutes at 54°C and 1 minute at 72°C; thereafter, to enhance the sensitivity, 22 cycles of 1 minute 95°C, 2 minutes at 54°C, and 1 minute at 72°C and a final extension step for 10 minutes at 72°C. The PCR amplification products were separated on 1.5% agarose and visualised using ethidium bromide. A 1kb DNA ladder (Gibco BRL- Life technologies, Paisley, UK) was used for length determination

2.5 *Streptococcus pneumoniae*

2.5.1 Culture of *Streptococcus pneumoniae*

Bacteria were obtained from clinical isolates at the Royal Free Hospital kindly provided by Prof S. Gillespie. Pneumococcal strains were confirmed by agglutination tests using specific antibodies and shown to be antibiotic sensitive. Encapsulated *S. pneumoniae* serotype 3 and non-encapsulated strain R36A were used for culture. Bacteria were grown in Brain Heart Infusion (BHI, Fisher Chemicals, Loughborough, UK), broth and incubated at 37°C, 5% CO_2 for 16 hours. Overnight cultures were harvested at 1000 g for 28 minutes and known concentrations of bacterial aliquots were stored at –70°C with 10% glycerol until required. Bacteria were diluted in CD-hybridoma medium and centrifuged at 2000g for 15 minutes. Pellets were resuspended in CD-hybridoma medium or PBS at the required concentration and used. Bacteria were considered to be in a logarithmic phase of growth curve since at approximately 24 hours the stationary phase was reached.

2.5.2 FITC labelling of *Streptococcus pneumoniae*

S. pneumoniae was cultured as explained above and harvested at 2000g in PBS. Approximate concentrations were calculated on the basis that OD_{600} of 1.0 is equivalent to 5 x 10^8 organisms/ml. *S. pneumoniae* was incubated with 3% (v/v) formaldehyde (Sigma- Aldrich, Gillingham, UK) at 37°C for 1 hour, then washed twice with PBS at 2000g for 5 minutes and then was resuspended in 1 ml 0.5mg/ml fluorescein isothiocyanate-FITC (Sigma - Aldrich, Gillingham, UK) for 1 hour at 37°C. After at least four washes with PBS, FITC- labelled pneumococci were maintained at 4°C and washed prior to use. No experiments were performed after 1 week of being labelled.

2.5.3 MBL binding to *Streptococcus pneumoniae*

1 x 10^6 of *Streptococcus pneumoniae* and *Cryptococcus neoformans* (provided by Microbiology Unit at LSHTM) fixed with 3% (v/v)-formaldehyde in the same way as *S. pneumoniae* (see section 2.5.2) were incubated with 10 μg/ml of MBL in Hank's Balanced Salt Solution (HBSS- Sigma – Aldrich, Gillingham, UK) with 10mM $CaCl_2$ for 1 hour at 4°C in falcon tubes. After two washes with HBSS, mouse biotinylated antibody to human MBL (Statens Serum Institute, Copenhagen, Denmark) diluted 1 in 100 μl (v/v) HBSS were added and incubated for 1 hour at 4°C. In some tubes, a mouse biotinylated isotype control (Jackson Laboratories, West Grove, Pennsylvania, USA) was added under the same conditions. Following two washes, streptavidin-FITC (BD Biosciences, Oxford, UK) diluted 1/100 in HBSS (100μl) was added and incubated for 1 hour at 4°C. After two more washes the cells were resuspended in 200 μl of HBSS and 30000 events were acquired by flow cytometry (FACS SCALIBUR- Becton Dickinson, Oxford, UK) and analysed using Cell Quest Software (Becton Dickinson, Oxford, UK).

2.5.4 CRP binding to *S. pneumoniae*

To 2 x 10^6 *S. pneumoniae* type 3, polyclonal rabbit antibody to human CRP (DakoCytomation, Ely, Cambridgeshire UK) diluted 1/50 (166 μg/ml) and 1/100 (83

μg/ml) in 100 μl HBSS containing 10mM $CaCl_2$ were added and incubated for 1 hour at 4°C. Two washes with HBSS were performed and then a FITC conjugated goat anti-rabbit antibody (Stratech Scientific, Soham Cambs, UK) diluted 1/100 in 100 μl HBSS was added and incubated for 1 hour at 4°C.

An irrelevant rabbit IgG antibody was used as an isotype control at the same concentrations specified (Stratech Scientific, Soham Cambs, UK).

After two more washes, at least 30000 cells were acquired by flow cytometry (FACS SCALIBUR- Becton Dickinson, Oxford, UK) and analysed using Cell Quest Software (Becton Dickinson, Oxford, UK)

2.6 Expression of receptors by neutrophils

2.6.1 Expression of L-selectin by neutrophils

Isolated neutrophils (obtained by both the Dextran and Histopaque methods, see section 2.2) were resuspended at 2 x 10^6/ml. 200 μl of neutrophils were added to 5-ml falcon tubes, were incubated in CD-hybridoma medium, in the presence or absence of 100 IU of IFNγ at 37°C for 8 hours. After 2 washes with PBS at 300g for 5 minutes, 5 μg/ml mouse antibody to human L-selectin (R&D Systems, Abingdon, UK) were added to each tube for 1 hour at 4°C in FACS buffer containing Hanks's Balanced Salt Solution (HBSS- Gibco BRL, Life technologies, Paisley, UK), 3% (v/v) FCS, 10mM HEPES, 5mM EDTA and 0.05% (w/v) sodium azide. A mouse IgG1 isotype control (provided by Dr John Raynes) was used at the same concentration for 1 hour at 4°C in FACS buffer. After two washes with PBS, a goat anti-mouse tetramethyl-rhodamine isothiocyanate (TRITC) conjugate (Sigma-Aldrich, Gillingham, UK) diluted 1/100 in FACS buffer was added for 1 hour at 4°C. At least 10000 events were acquired for FACS analysis.

2.6.2 Expression of FcγRI

Isolated neutrophils (obtained by both Dextran and Histopaque methods) were resuspended at 2 x 10^6/ml in CD-hybridoma medium. 200 μl of neutrophils were added to 5-ml falcon tubes, and were incubated overnight in the presence or absence

of 100 IU of IFNγ at 37°C. After 2 washes with PBS at 300g for 5 minutes, 0.4 μg/ml of phycoerythrin (PE) conjugated IgG1 mouse antibody to human FcγRI (CALTAG Laboratories, Burlingame, CA, USA) was added to each tube for 1 hour at 4°C. A PE mouse IgG1 isotype control (CALTAG Laboratories, Burlingame, CA, USA) (in 100μl of FACS buffer) was used at the same concentration and under similar conditions. At least 10000 events were acquired for FACS analysis and analysed by Cell Quest Software (Becton Dickinson, Oxford, UK).

2.6.3 Expression of FcγRIIB

Neutrophils isolated by the dextran method were resuspended at 2 x 10^6/ml in CD-hybridoma medium. 200 μl were added to 5 ml falcon tubes and after 1 wash with PBS were resuspended for 30 minutes at RT in 100 μl of permeabilising buffer containing Hanks's Balanced salt solution (HBSS- Gibco BRL, Life technologies, Paisley, UK) 0.5% (w/v) bovine serum albumin (BSA), 0.5%(w/v) saponin and 0.05% (w/v) sodium azide. Cells were centrifuged and resuspended in 100 μl of FACS buffer.

0.4 μg of an IgG affinity purified goat polyclonal antibody raised against the carboxy terminal portion of the human FcγRIIB (C-20) (sc-12815 Santa Cruz Biotechnology, Inc., Loughborough, UK) was added for 1 hour at 4 °C in 100 μl of permeabilising buffer. In some experiments, 0.4 μg of an IgG affinity purified goat polyclonal antibody against an aminoterminal portion of human FcγRIIB (N-17) (sc-12812 Santa Cruz Biotechnology, Inc., Loughborough, UK) was used. The same amount of an irrelevant goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Loughborough, UK) was used as an isotype control. To examine specificity 5, 10, 20 and 25 μg of an inhibitory peptide anti-C-20 (Santa Cruz Biotechnology, Inc., Loughborough, UK) was used in some experiments.

2.7 Functional activities of neutrophils

2.7.1 Culture of Neutrophils with APPs

Purified neutrophils, either from HH or RR donors, were primed or not (as a control) by using 100 IU/ml of IFNγ for at least 12 hours at 37°C. Human IgG1 (10 μg/ml) was added to some wells at 4°C for 30 minutes. Then 100 μl of a $2x10^5$ suspension of neutrophils were added to each well of a 96-well culture plate, then 50 μl of either type 3 or R36A *S. pneumoniae* were added to obtain a final ratio of 30:1 (bacteria:cell).

Finally, 50 μl of CRP at three different concentrations (100, 50 or 10 μg/ml), or MBL at two different concentrations (30 or 1μg/ml) were added in triplicate. After that, neutrophils were cultured for a further 8 hours at 37°C, centrifuged at 800g for 5 minutes and samples collected for ELISA determinations. Where stated 10 ng/ml Polymixin B (Sigma-Aldrich, Gillingham, UK) was used in order to reduce the effects of possible endotoxin contamination.

2.7.2 Production of cytokines by neutrophils

2.7.2.1 Determination of TNFα

TNFα was determined by an ELISA procedure. 96-well flat bottom plates (Immulon 2HB, Dynex technologies, UK) were coated with 50 μl of 2μg/ml mouse IgG2b anti-human TNFα monoclonal antibody (BD Biosciences, Oxford, UK) in 0.1M di-sodium hydrogen orthophosphate pH 9.0 (Na_2HPO_4)(BDH Laboratory supplies, Poole, England) and incubated overnight at 4°C.

Then, plates were washed 4 times with PBS containing Tween20 0.05% v/v (PBST). Wells were blocked by adding 200μl of PBST/10% (v/v) FCS for 1 hour at RT. Standard curves were prepared in duplicate, by adding 50 μl of dilutions of 2 ng/ml Human TNFα (National Institute for Biological Standards and Control, UK). Serial

doubling dilutions were made thereafter down to 31.25 pg/ml. Samples were also added in duplicate and left overnight at 4°C.

After 4 washes with PBST, 50 µl of IgG2b biotinylated mouse anti-human TNFα monoclonal antibody (BD Biosciences, Oxford, UK) were added and the tubes left for 2 hours at RT. Following extensive washing with PBST, 50 µl of horseradish peroxidase (HRP)-streptavidin (Dako SA, Glostrup, Denmark) were added for 1 hour at RT. After 3 washes with PBST and 3 with PBS, 50 µl of tetramethylbenzidine-TMB (Sigma-Aldrich, Gillingham, UK) in citrate buffer pH 4.5 (0.1M Citric acid and 0.1 M Na_2PO_4 diluted 2 volumes of acid plus 3 volumes of base) plus H_2O_2 were added, as a substrate for 30 minutes at RT. The reaction was stopped with 12.5 µl of 2M sulphuric acid and plates read at 450nm and control wavelength 490 nm in an ELISA reader (MRX Microplate Reader, Dynex technologies, Southampton, UK). The assay sensitivity was 50 pg/ml.

2.7.2.2 Determination of IL-8

IL-8 was determined by an ELISA procedure. 96-well flat bottom plates (Immulon 2HB, Dynex technologies, Southampton, UK) were coated with 50 µl of 2µg/ml mouse IgG2b anti-Human IL-8 monoclonal antibody (BD Biosciences, Oxford, UK) diluted in 0.1M di-sodium hydrogen orthophosphate pH 9.0 (Na_2HPO_4)(BDH Laboratory supplies, Poole, England) and incubated overnight at 4°C.

Then plates were washed 4 times with PBST, wells were blocked by adding 200µl of PBST 10% (v/v) FCS for 1 hour at RT. Standard curves were performed in duplicate by adding 50 µl of human rIL-8 (range 2 ng/ml to 31 pg/ml) (National Institute for Biological Standards and Control, UK), then samples were also added in duplicate and left overnight at 4°C.

After 4 washes with PBST, 50 µl of IgG2b biotinylated mouse anti-human IL-8 monoclonal antibody (BD Biosciences, Oxford, UK) were added and left for 2 hours at RT. Then 50 µl of HRP-Streptavidin (Dako SA, Glostrup, Denmark) were added for 1 hour at RT and finally 50 µl of TMB (Sigma-Aldrich, Gillingham, UK) as

described in section 2.7.2.1 for 30 minutes. The reaction was stopped with 12.5 µl of 2M sulphuric acid and plates read at 450nm with a control wavelength at 490nm in a ELISA reader (MRX Microplate Reader, Dynex technologies, Southampton, UK). The assay sensitivity was 31.25 pg/ml.

2.7.2.3 Intracellular cytokines

Neutrophils were resuspended at 5 x 10^6/ml in CD-Hybridoma medium. Different stimuli were added: PMA at 5 µM (Sigma-Aldrich, Gillingham, UK), fMLP 100 nM (Sigma-Aldrich, Dorset, UK), LPS at 1 µg/ml (Sigma-Aldrich, Gillingham, UK) for 1 hour at 37°C. After 2 washes with a solution containing Hanks's balanced salt solution (HBSS- Gibco BRL, Life technologies, Paisley, UK), 3% (v/v) FCS, 10mM HEPES, 5mM EDTA and 0.05% (w/v) sodium azide (Buffer A), 1 µl per condition of Brefeldin A (previously diluted at 1 mg/ml in 70% ethanol) was added to 100 µl. Cells where further incubated for 4 h at 37°C and then cells were fixed with 1% (v/v) paraformaldehyde. Cells were washed with Buffer A at 300g for 2 minutes.

Further incubations were carried out in the dark. Cells were permeabilised with a solution containing HBSS, 0.5% w/v BSA, 0.5% (w/v) saponin and 0.05% (w/v) sodium azide (Buffer B), by incubating them in 200 µl of buffer B for 2 minutes at RT. Biotinylated antibodies to human IL-8 (BD Biosciences, Oxford, UK) or human TNFα (BD PharMingen, UK) were diluted to 5 µg/ml in Buffer B, and 100 µl of each were added to each tube and the mixtures incubated for 30 minutes at RT. Then, after 2 washes with Buffer B, 5 µg/ml of Streptavidin-FITC conjugate (BD Biosciences, Oxford, UK) was added to each condition and incubated for 30 minutes at RT. Finally, cells were washed twice with Buffer A and resuspended to 200 µl with Buffer A for acquisition. At least 20000 events were acquired by FACS (FACS SCALIBUR, Becton Dickinson, Oxford, UK). As isotype controls an irrelevant biotinylated IgG1 mouse antibody was used at the same concentrations as described for antibodies to IL-8 and to TNFα.

2.7.3 NADPH oxidase activity in neutrophils

2.7.3.1 Flow Cytometric analysis of respiratory burst by isolated neutrophils

This procedure was based on a previously described technique for NADPH oxidase analysis using human neutrophils by FACS (Emmendorffer *et al.*, 1990). Isolated neutrophils were counted and resuspended at 2 x 10^6 cells/ml in HBSS (Sigma – Aldrich, Gillingham, UK) then cultured overnight with or without IFNγ (100 IU/ml). 100 µl aliquots of cells were incubated in plastic falcon tubes with 100 µl of stimuli (CRP at final concentrations of 100, 50 and 10 µg/ml alone or with Pneumococci Serotype 3 at a ratio 30:1); PMA at 1 µM was used as a positive control diluted in PBS for 20 minutes at 37°C.

Then, 50 µl of 100 µM of dihydrorhodamine 123 (DHR- Calbiochem, Nottingham, UK) diluted in PBS were added and cells left at 37°C for further 5 minutes.

The reaction was stopped with 200 µl PBS-0.02% EDTA and washed once with PBS. The cells were fixed with 200 µl of cold 1% paraformaldehyde (PFA) at 4°C and stored at 4°C until read.

Cells were acquired by flow cytometry (FACS SCALIBUR, Becton-Dickinson, Oxford, UK) and analysed by Cell Quest software (Becton–Dickinson, Oxford, UK). At least 30000 events were counted per sample.

2.7.3.2 Flow Cytometric analysis of respiratory burst by neutrophils maintained in whole blood

Some experiments were performed using whole blood from donors as follows according to a procedure described previously (Richardson *et al.*, 1998). Blood was collected into 50 ml falcon tubes containing Citrate Dextrose solution (ACD-Sigma-Aldrich, Gillingham, UK), as an anticoagulant at a ratio 1: 5 (ACD: Blood). 100 µl of blood were transferred to plastic falcon tubes and different stimuli were added according to the assay performed, e.g. 5 µM of PMA, purified or recombinant CRP, pneumococcus serotype 3 for 20 minutes at 37°C. 50 µl of 100 µM DHR were then added and cells incubated for a further 10 minutes at 37°C for analysis of NADPH oxidase activity of neutrophils.

Then FACS lysing solution (BD Biosciences, Oxford, UK) was added during 20 minutes at 37°C to lyse red blood cells. The cells were centrifuged twice at 1000g for 2 minutes and then fixed with PFA1% (v/v) at 4°C. At least 20000 events were acquired by flow cytometry (FACS SCALIBUR – Becton Dickinson, Oxford, UK) and data analysed using Cell Quest software (Becton Dickinson, Oxford, UK).

2.7.3.3 H_2O_2 release from human neutrophils

The Amplex Red Hydrogen Peroxide Assay Kit (A-12212 – Molecular probes) was followed according to the instructions from the manufacturer based on the protocol of Mohanty *et al.,* (1997). Briefly, a reaction mixture containing 50μM of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), which becomes fluorescent upon H_2O_2 oxidation (resorufin), 1 U/ml of horseradish peroxidase (HRP) to catalyse H_2O_2 oxidation and stimuli (PMA at a final concentration of 5μM, *S. pneumoniae* at 4.5 x 10^5 and in some cases CRP at final concentrations of 1, 5, 10, 50 and 100 μg/ml) in 180 μl of CD-hybridoma medium was added to a 96-well plate.

The reaction mixture was prewarmed for 10 minutes at 37°C, then, 1.5 x 10^4 isolated neutrophils in 20 μl of CD-hybridoma medium were added to make up a total volume of 200 μl. Fluorescence was determined using an excitation of 560 nm and emission detection at 590 nm. Readings were taken each 15 minutes for 2 hours in a microplate spectrofluorometer reader (Spectra MAX Gemini, Sunnyvale, USA).

2.7.3.4 Superoxide production by neutrophils

Isolated neutrophils were resuspended at 2 x 10^6/ml and 50 μl per well were added to a 96-well plate. 30.5 μl of stimuli were used as follows; PMA at a final concentration of 5μM as a positive control and recombinant CRP at 1, 5, 10, 50 and 100 μg/ml. In some wells superoxide dismutase (SOD) (Sigma–Aldrich, Gillingham, UK) at 300 Units/ml was used as negative control. Then, 75μM cytochrome c (Sigma –Aldrich, Gillingham, UK) in 12.5 μl was added and CD –hybridoma medium used to make a total volume of 250 μl in each well.

Absorbances were determined each 15 minutes up to 2 hours at 550nm in a microplate spectrofluorometer reader (Spectra MAX Gemini, Sunnyvale, USA). The amount of reduction of cytochrome c was calculated by subtracting the OD obtained with SOD from the one obtained with each sample, using a molar extinction coefficient of 21.1 x 10^{-3}M and a light path of 0.66 cm for a final volume of 250µl. as follows:

$$\text{nmol}/10^5 \text{ cells} = \left[\frac{\text{(sample OD} - \text{SOD OD)}}{21.1} \times 0.66\right] \times 1000$$

2.7.4 Phagocytosis

2.7.4.1 Phagocytosis by transfected Cos cells

2.7.4.1.1 Transfection of Cos cells

Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 2 mM glutamine (Life Technologies, Paisley, UK), 100 IU/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Paisley, UK), 0·015 mg/ml gentamicin (Sigma-Aldrich, Gillingham, UK) and 10% fetal bovine serum (Sigma-Aldrich, Gillingham, UK). Cells were plated at 4×10^5/ml in 60-mm Petri dishes to reach 50% confluence overnight.

The simian virus 40-based expression vector CDM was used for the transient expression of the cDNAs of all clones in Cos-7 cells using the diethylaminoethyl-dextran method (Allen and Seed, 1989). The cDNAs (5 µg/3 ml Petri dish) used for transfection were FcγRI, γ-chain and Syk (obtained from Professor Janet Allen, Department of Medicine and Therapeutics, Division of Biochemistry & Molecular Biology, The University of Glasgow, UK) FcγRII RR and FcγRII HH (obtained from Dr Patrick Harrison Department of Physiology, University College Cork, Ireland). FcγRII R tailers mutant was generated from FcγRII R by PCR to

incorporate a terminal phenylalanine to replace Tyr at position 205 (Rachel Gregory, LSHTM). All experiments were performed 2 or 3 days post-transfection, when surface expression was maximal. This work was performed by Dr K. Bodman-Smith as previously described (Bodman-Smith *et al.*, 2002).

2.7.4.1.2 Phagocytosis of *S. pneumoniae* by transfected Cos cells

FITC-labelled *Streptococcus pneumoniae* serotypes 3 and 2 strain R36A (see section 2.5.2) were opsonised either with pCRP or rCRP at 30 μg/ml in the presence of 5mM $CaCl_2$ or with 10% normal human serum (NHS-Sigma – Aldrich, Gillingham, UK) as a positive control for 1 hour at 4°C. Bacteria were washed extensively with HBSS (Sigma – Aldrich, Gillingham, UK). Opsonised and non-opsonised bacteria were allowed to remain in contact with transfected Cos cells at a ratio of 30:1 in HBSS in the presence of 0.5mM $CaCl_2$ for 2 hours at 37°C. Cells were transferred into falcon tubes and were washed with HBSS in the presence of 1%BSA. Cells were then fixed with 2%(w/v) PFA and were acquired by Flow cytometry (FACS SCALIBUR, Becton-Dickinson, Oxford, UK).

2.7.4.2 Phagocytosis by neutrophils

Isolated neutrophils were resuspended in CD-hybridoma medium at 2 x 10^6 cells/ml, were incubated with FITC-labelled pneumococci (see section 2.5.2), at different ratios 10:1, 30:1 and 100:1 (bacteria:cells) at 37°C for 30 minutes. In another experiment cells were divided into two groups, incubated with pneumococci at a ratio of 30:1 at 4°C and compared with a group of cells warmed to 37°C. In some experiments neutrophils were incubated with FITC-labelled pneumococci and 30μg/ml of CRP, in the presence or absence of 1 μM Cytochalasin D (Sigma – Aldrich, Gillingham, UK) at 37°C for 30 minutes.

Neutrophils from both HH and RR donors were obtained and incubated with pneumococci at a ratio of 30:1 for 30 minutes at 37°C in the presence of pCRP or rCRP at 0, 1, 5, 10, 50 or 100 μg/ml.

In all experiments, phagocytosis was stopped with cold PBS; after two washes with cold PBS at 300 g for 5 minutes, cells were fixed with 1% PFA for 30 minutes and at least 20000 events were acquired by Flow cytometry (FACS SCALIBUR, Becton-Dickinson, Oxford, UK). Phagocytosis was calculated by obtaining differences in the mean fluorescent intensity (MFI) of cells at 37°C from the one obtained with cytochalasin D.

2.7.4.3 Phagocytosis by neutrophils maintained in whole blood

A similar procedure to that described in section 2.7.3.2 was used to examine FITC-labelled bacteria for phagocytosis in whole blood in the presence or absence of different concentrations of added pCRP or rCRP. A total volume of 200 µl per tube was used in all assays.

2.7.4.4 Phagocytosis by Peripheral Blood Mononuclear Cell - derived macrophages

PBMCs resuspended in RPMI were allowed to adhere for 2 hours at 37°C in plastic slides in serum free medium and then cultured for 6 days in RPMI with 10% FCS at 37°C. 4 x 10^4 macrophages per well were incubated in contact of FITC labelled *Streptococcus pneumoniae* type 3 and strain R36A at a ratio 30:1 in the presence of absence of pCRP and rCRP at 30 µg/ml or in the presence of 10% NHS as a control for 1 hour at 4°C. After two washes with PBS, cells were fixed with 300 µl of methanol for 10 minutes at RT and then 50µM ethidium bromide was added to stain nuclei of macrophages for 20 minutes at RT. Phagocytosis was visualised by immunofluorescence.

2.7.4.5 Visualization of phagocytosis of *S.pneumoniae* by neutrophils by confocal microscopy

Neutrophils were isolated and resuspended at 2 x 10^6/ml. 100 µl of neutrophils were incubated with 200 µl of HBSS containing either FITC-labelled *S.pneumoniae* serotype 3 alone or pre-opsonised with CRP at 30 µg/ml or 10% NHS at a ratio 1:30

(neutrophil: pneumococci) for 30 minutes at 37°C on glass slides to allow adherence of neutrophils. Then, phagocytosis was stopped with cold HBSS containing 1% (w/v) BSA and washed three times. The cells were fixed with 300 µl of methanol and were washed three times with HBSS containing 0.5% (w/v) saponin to permeabilise the cells. 300 µl of BOPIDY® 650/665 phalloidin (Molecular probes, Leiden, The Netherlands) to detect actin diluted 1/100 in HBSS-saponin solution were added and incubated for 45 minutes at RT. Finally, 50µM ethidium bromide (EB) was added for 20 minutes at RT. Phagocytosis was visualised by confocal microscopy in collaboration with Rachel Gregory

2.8 Binding of CRP and SAP to FcγRs by surface plasmon resonance

2.8.1 Preparation of samples

Recombinant soluble FcγRIIA-RR, RIIB and RIIIB –NA2 were produced in *E.coli* strain BL21/DE3 (Sondermann and Jacob, 1999) and were obtained from Professor P Sondermann (Max-Planck Institute für Biochemie, Abteilung Strukturforschung, Martinsreid, Germany). Samples were dialysed first against 0.1M $NaHCO_3$ at 4°C. Biotinylation was performed as follows: a stock of 8mM Biotin-N-hydroxy-succinimide (Biotin-NHS) (Sigma – Aldrich, Gillingham, UK) was prepared and later diluted 50-fold into milliQ H_2O. 3 µl of diluted biotin-NHS were added to each FcγR and left at 25°C for 1 hour (ratio biotin: FcγR, 0.8:1). Samples were dialysed against 100 ml of 150mM NaCl 10 mM HEPES pH 7.4 at 4°C, then against a further 100 ml of the same solution plus 0.004% (w/v) sodium azide overnight at 4°C. The final concentration of the FcγR samples was 0.6µg/ml.

2.8.2 Offering Fc receptor to CRP bound to PCh immobilised on a CM5 chip

On a CM5 sensorchip, 100 µM ρ–aminophenyl-PCh was immobilised in flow cell 2 whereas flow cell 1, which was blocked with 1M ethanolamine, pH 8.5, was used as a control. Flow cell 2 was activated with 35 µl 0.05 M N-hydroxysuccinimide and 35 µl of 0.2M N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide at 5 µl per minute. Then, 30 µl 100 µM ρ–aminophenyl-PCh in 0.1 M boric acid – NaOH, pH 8.4 were

injected and remaining activated groups were blocked with 30 µl 1M ethanolamine-HCl pH 8.5. Flow cell 1 was activated in the same way and it was blocked with ethanolamine.

Then different concentrations of both rCRP and pCRP from 0.1 µg/ml up to 10 µg/ml were injected. Finally, different concentrations (100, 50, 20 and 10 µg/ml) of FcγRIIA or FcγRIIB were injected in a buffer containing 0.15M NaCl, 0.5mM $CaCl_2$, 0.05% (v/v) Tween 20, 0.01M HEPES, 0.1 M boric acid NaOH, pH 8.4 at a flow rate of 10 µl per minute.

2.8.3 Offering CRP to biotin-Fc receptors immobilised on a streptavidin chip

On a streptavidin (SA) sensorchip (BIACORE, Stevenage, UK) biotinylated FcγRIIA-RR allele, FcγRIIB and FcγRIIIB –NA2 were immobilised to obtain about 500 RU in flow cells 2, 3 and 4 respectively, whereas flow cell 1 was left as blank. Possible remaining free sites on the chip were blocked with 20 µl of 0.36 mM biotin. Then 10 µl of different concentrations from 0.2 µg/ml to 50 µg/ml of rCRP or pCRP were injected at a flow rate of 10µl/min in the presence of 0.5 mM $CaCl_2$ in a buffer containing 10mM HEPES, 100 mM NaCl and 0.005% (v/v) of Tween20. Binding was analysed using a BIACORE 3000 and BIA evaluation 2.1 software (BIACORE, Stevenage, UK). 10 µl of SAP at 0.1, 1, 10 and 30 µg/ml were also injected using the same conditions since it was reported that SAP binds to mFcγRI and mFcγRIII but not to mFcγRII (Mold *et al.*, 2001). Sensitivity of the Biacore was 1000 RU equivalent to 1 ng/mm^2.

2.9 Statistical analysis.

The software GraphPad Prism® 3.03 version was used for statistical analysis. The unpaired Mann Whitney test was used for most analyses. p values, when applicable, were considered significant when lower than 0.05. In all cases * represents $p<0.05$, ** represents $p<0.01$ and *** $p< 0.001$.

3. RESULTS

3.1 Purification of Neutrophils

There are different techniques to isolate neutrophils, based mainly on ways to remove red blood cells by using their property to aggregate to each other thus becoming heavier than other cells. However, these methodologies affect the viability and activation of neutrophils which may be of crucial importance for particular assays where the function of neutrophils is being analysed.

The activation state of neutrophils was assessed following different methods of isolation: first, a histopaque based method that uses two densities of histopaque 1.166 and 1.077, which allows simultaneous separation of a layer of mononuclear cells at the upper interface and PMN cells at a lower interface. Red blood cells are separated at the bottom of the tube. Second, a dextran method, in which leukocyte – enriched plasma is obtained after incubation of blood with dextran, following which mononuclear cells are separated from PMN by centrifugation of the plasma on histopaque (ρ=1.077). Both of these methodologies gave a purification of more than 95% neutrophils which was confirmed by Wright-Giemsa staining (figure 3.1).

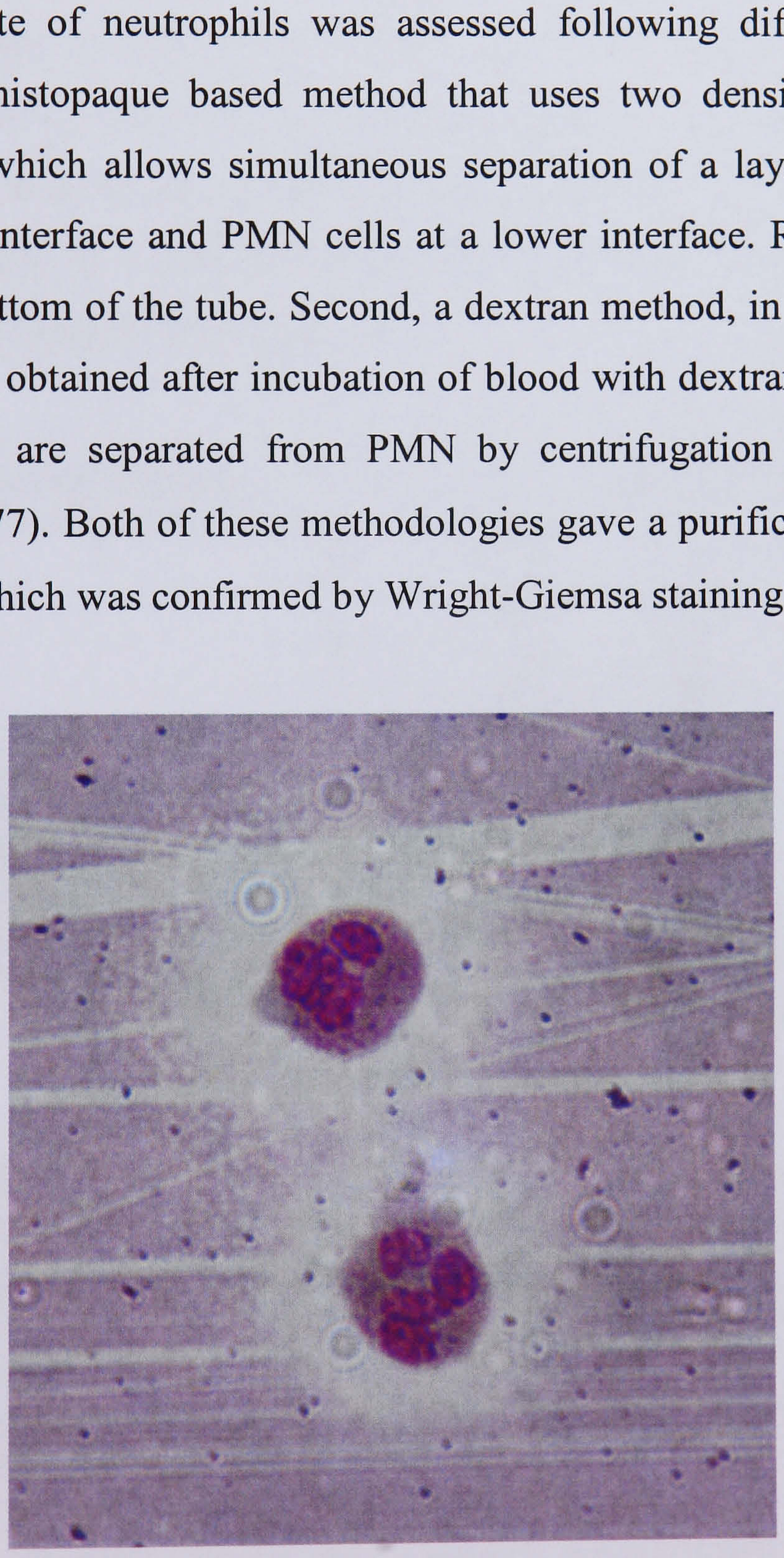


Figure 3.1. Isolated human neutrophils. Human blood from healthy donors was treated with dextran to remove red blood cells and then neutrophils were obtained through centrifugation with histopaque 1.077. Separated cells were visualised by staining a smear on glass slides with Wright - Giemsa.

3.2 State of activation of neutrophils after isolation procedure

The most commonly used separation methods for neutrophils involve density gradient centrifugation, with additional steps to remove contaminating red blood cells. Such gradients are usually discontinuous and are generally based on Percoll (colloidal silica coated with polyvinylpyrrolidone to reduce toxicity) or on Ficoll (a sucrose polymer); Ficoll may be combined with sodium diatrozate (Hypaque) or with Isopaque to increase density. A method to reduce red cell contamination is used in combination with gradient centrifugation; either sedimentation enhanced by dextran (occasionally gelatine or hydroxyethyl starch are used in place of dextran) and red cell lysis for remaining red blood cells with ammonium chloride or hypotonic saline are used. We have used the combination of two densities (1.116 and 1.077) of Ficoll-Isopaque (Histopaque) or a combination of dextran and then Ficoll-Isopaque (1.077) to remove contaminating mononuclear cells.

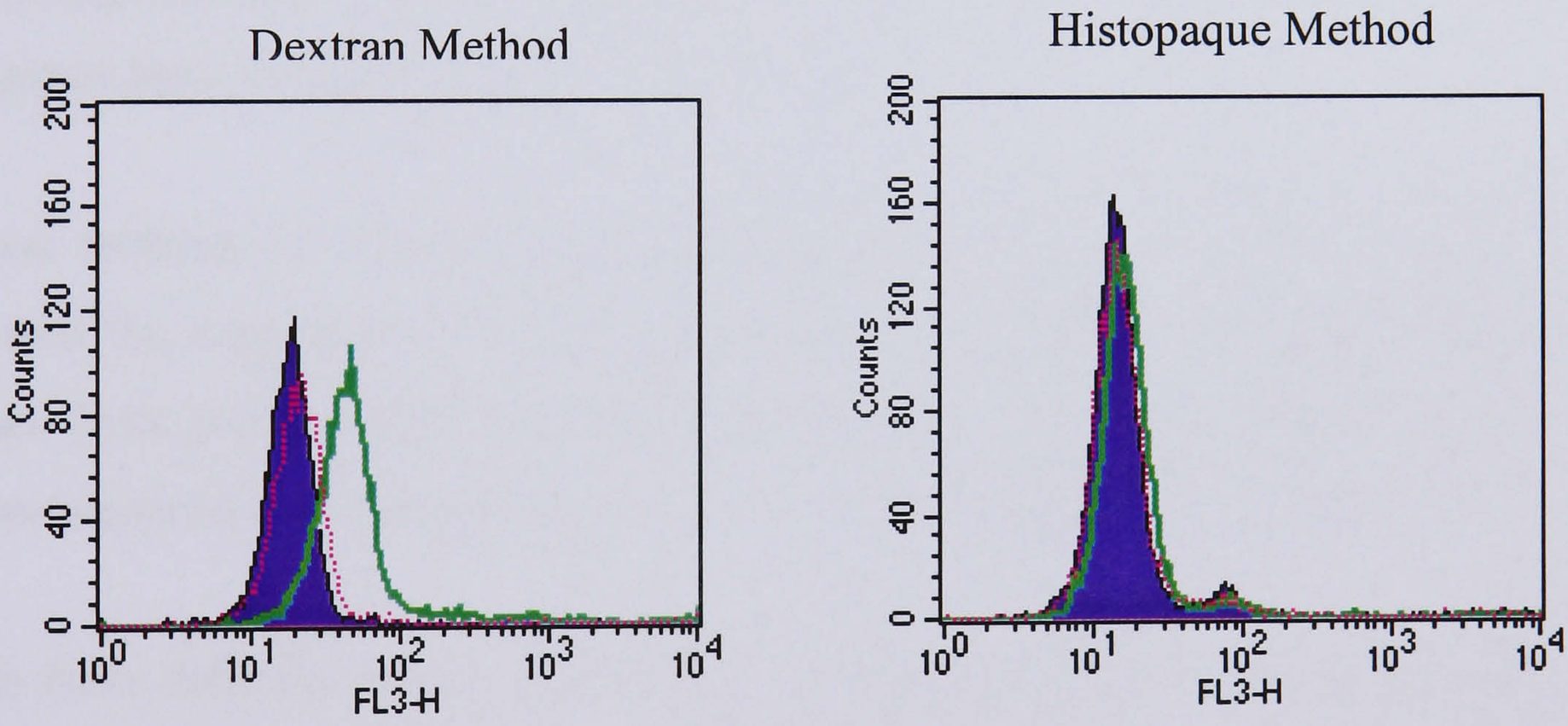


Figure 3.2. L-selectin expression on neutrophils. L-selectin on neutrophils obtained by the Dextran- or Histopaque-methods was detected with a mouse IgG1 monoclonal anti-human L-selectin antibody ——. A mouse IgG1 isotype control antibody ····· was used in both cases and compared with cells with no antibody ■. To obtain fluorescence a goat- anti mouse FITC conjugate was used. 10000 events were acquired and data was analysed using Cell Quest software. Similar results were obtained in two different experiments.

Neutrophils, when activated, change the expression of molecules on the membrane, L-selectin is shed, whilst integrins are upregulated, so if a neutrophil expresses high levels of L-selectin it is not yet fully activated. This property has been used to

analyse the state of activation of neutrophils isolated by both histopaque- and dextran-methods. Neutrophils obtained with the dextran-method showed higher expression of L-selectin than those isolated with two densities of histopaque (Figure 3.2).

Neutrophils express molecules that allow them to bind and phagocytose non-opsonised- and opsonised- particles. In this way, CR3 which binds to C3b, FcγRI and FcγRIIA which interact both with CRP and with monomeric IgG and IgG-immune complexes respectively are therefore particularly important for micro-organism clearance. On the other hand, neutrophils have low amounts of FcγRI on their membrane, but upon activation the expression increases exponentially with little alteration of the constitutive FcγRIIA. Many substances are able to activate neutrophils and induce FcγRI expression, among them IFNγ and GM-CSF have particularly potent activity. Expression of FcγRI can be visualised by indirect immunofluorescence, but for quantitative purposes flow cytometry is more appropriate because of its reliability and reproducibility.

Because methods of isolation of neutrophils can activate neutrophils as well, we compared the expression of FcγRI following isolation with either histopaque and dextran. Cells purified using dextran had less FcγRI (figure 3.3). For these reasons dextran separation was the technique of choice to isolate neutrophils in this study.

Serum from different species might also activate neutrophils, may contain MBL, CRP or SAP and therefore might alter the outcome or the reproducibility of the methods, for that reason we selected a medium that did not contain serum from any origin. CD-hybridoma® a serum-free medium which supports growth of hybridoma cells lines was selected to avoid the use of animal serum.

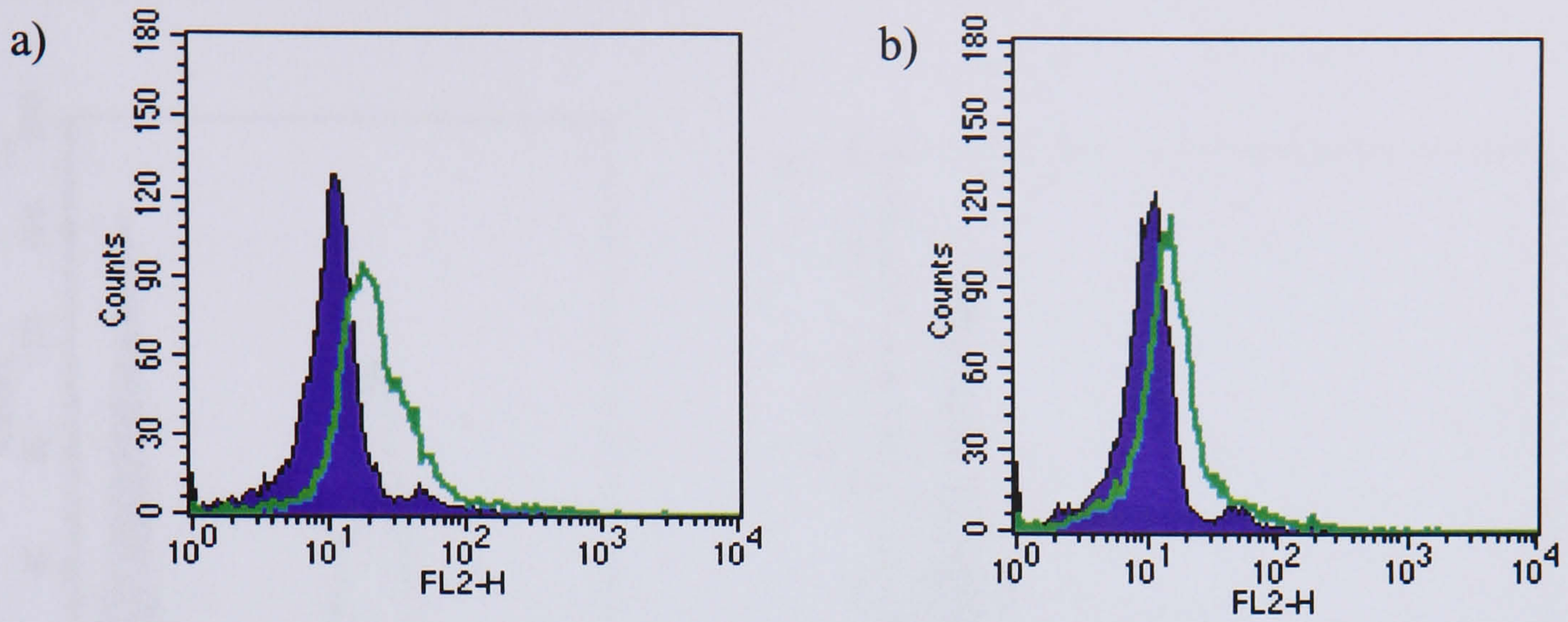


Figure 3.3. Expression of FcγRI in isolated untreated neutrophils. FcγRI expression was analysed in fresh neutrophils isolated by either two different histopaque densities (panel a) or Dextran (panel b) by adding a PE-conjugated mouse-IgG1 anti-human FcγRI antibody (green histogram) and compared with a PE-IgG1 mouse isotype control (filled histogram). This is a typical histogram from three different experiments showing similar results.

3.3 Apoptosis of neutrophils

Neutrophils are short lived cells, during normal situations from the time they abandon the bone marrow they usually last up to 24 hours. One of the explanations for this phenomenon is that neutrophils are programmed to die without any stimulus that "rescues" them from apoptosis. IFNγ, GM-CSF, LPS are stimuli that are able to have anti-apoptotic effects on neutrophils.

Apoptosis in its early stages is characterised by an exchange of phospholipids on the external membrane which allows phosphatidylserine to increase its expression. This is then used to identify apoptotic cells by binding to Annexin V, which can be associated with a fluorescent compound such as FITC. At later stages of apoptosis endonucleases of the cell are activated. These enzymes partially digest DNA which also can be detected in different ways, one of which labels DNA fragments at the 3' OH ends with TdT and biotinylated nucleotides which can be detected by Streptavidin-Fluorescein a technique known as Terminal deoxynucleotidyl transferase -mediated biotinylated deoxyuridine-triphosphate nick-end labeling (TUNEL).

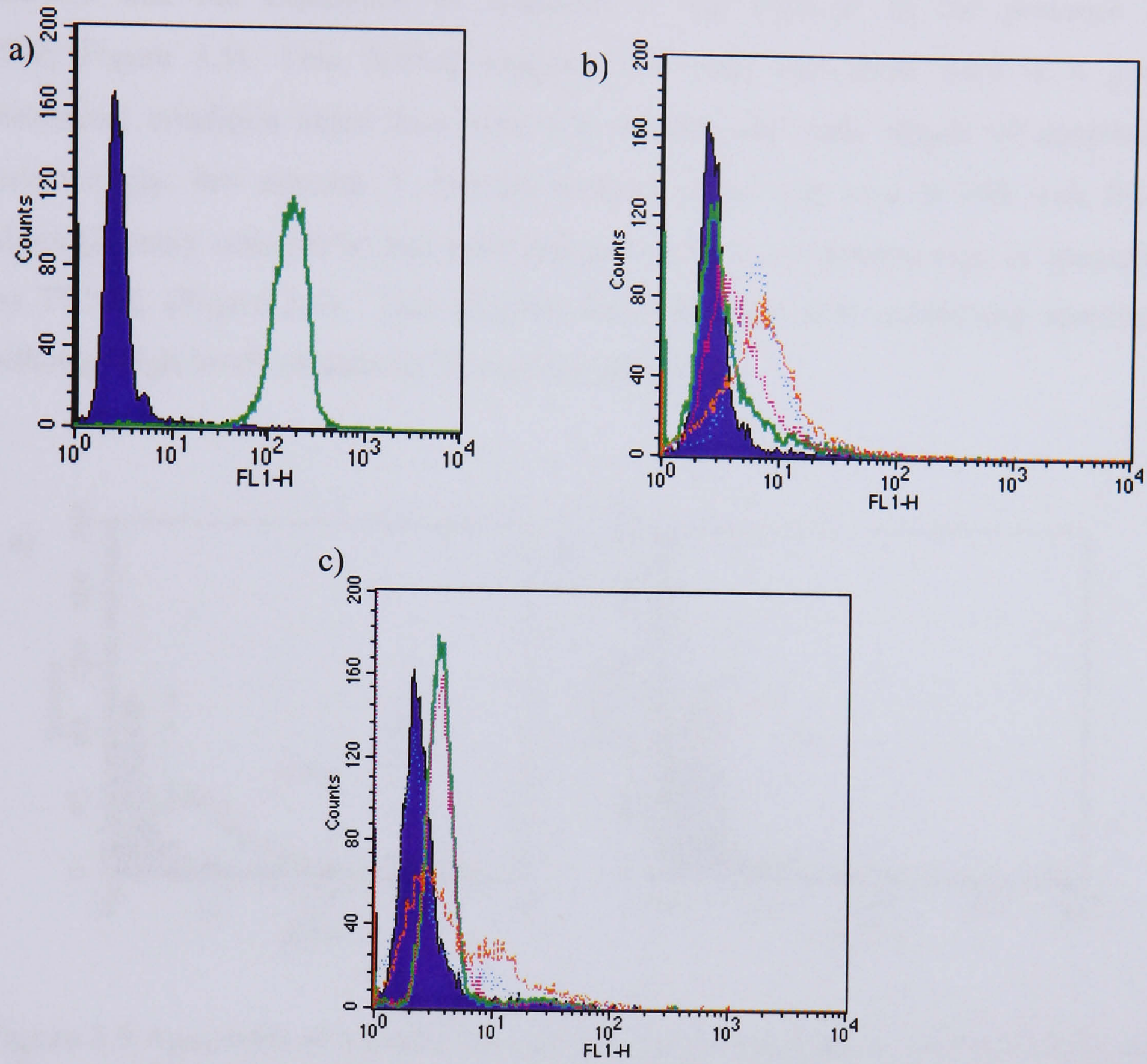


Figure 3.4. Apoptosis of neutrophils determined by TUNEL (Section 2.3.2). 1 x 10^6 neutrophils per test were incubated in serum free CD-hybridoma medium. In panel a) negative and positive controls (section 2.3.2) can be observed, in panel b) apoptosis was evaluated at 0 (filled histogram), 1 —— , 4 ····· , 18 hours····· and 24 hours—· in the absence of IFNγ and panel c)shows an identical study in the presence of IFNγ (100 IU/ml). Similar results were obtained in two separate experiments using two different donors.

We analysed neutrophils in apoptotic late stages by TUNEL during the first 24 hours. In Figure 3.4, panel b, it can be observed that apoptosis increases with time. In the absence of IFNγ, cells were counted at 24 hours using a hemocytometer and 50% of cells were lost during this period of time. In the presence of IFNγ TUNEL was less positive at most time points (panel c) and at least 60% of cells were still viable. It is clear, that IFNγ decreased apoptosis on neutrophils at least in the first 24 hours. No functional experiments were carried out later than 24 hours.

This result was correlated with the binding of Annexin V at 1, 4 and 24 hours which showed that the expression of Annexin V was reduced in the presence of IFNγ (Figure 3.5). This finding suggests that cells still alive were in a good functional condition since they were not entering the early stages of apoptosis. Interestingly, few annexin V strongly positive cells were seen at 24h with IFNγ although many cells (40%) had been lost and some were showing sign of apoptosis by TUNEL (Figure 3.4). This suggests that cells were still undergoing apoptosis although high levels of annexin V were not observed.

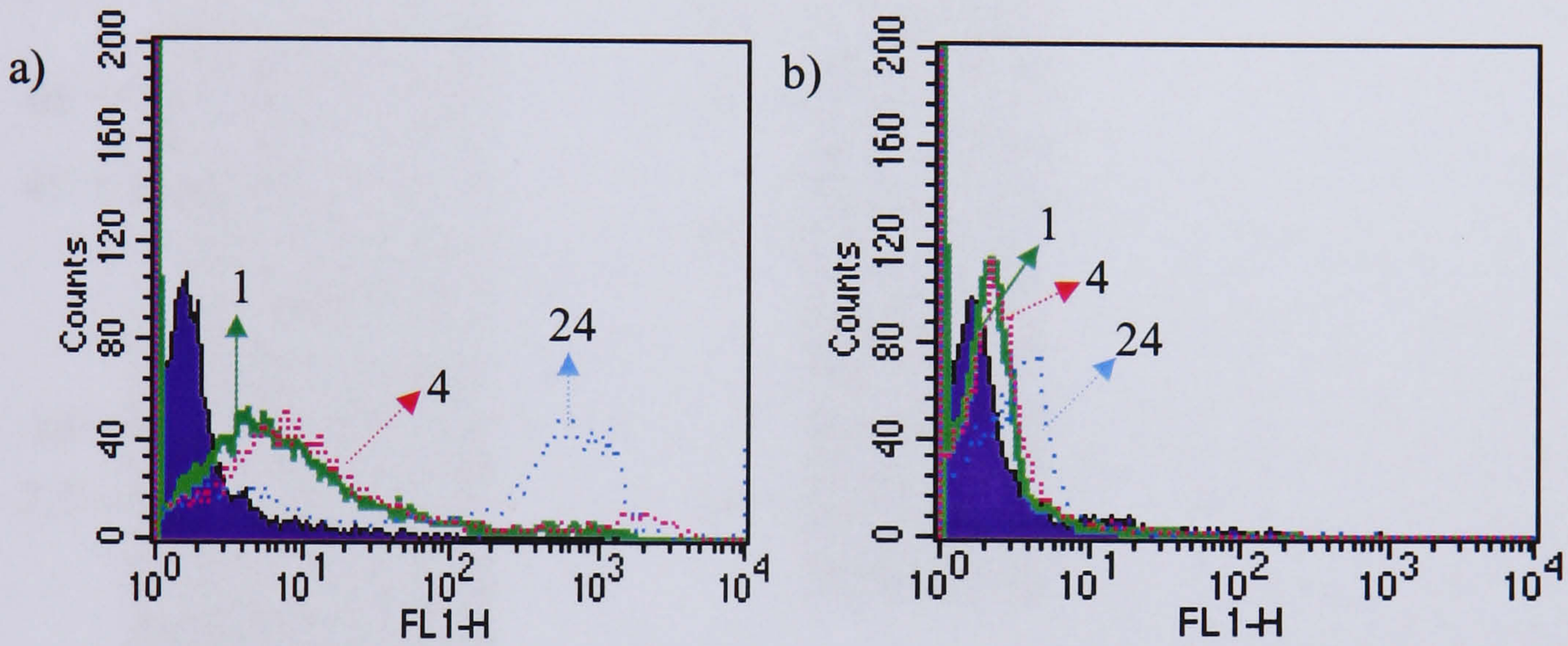


Figure 3.5 Apoptosis of neutrophils determined by Annexin V (section 2.3.1). 1 x 10^6 neutrophils per condition were incubated in the absence (panel a) or presence (panel b) of IFNγ 100IU/ml. Annexin V binding was analysed at 0 (filled histograms), 1 (green), 4 hours (red) and 24 hours (blue). Similar results were obtained in two separate experiments using two different donors.

3.4 Role of MBL in pneumococcal infections and interaction with neutrophils

3.4.1 Purification of MBL

MBL was obtained from the plasma of patients undergoing plasmaphoresis for acute inflammatory conditions as described in section 2.1.1 (Figure 3.6). MBL was the major protein component present as shown by ELISA and immunoblotting as well as protein staining which revealed a major band at 31 kDa.

3.4.2 Determination of MBL in some children with recurrent infections

Initially and in connection with a study examining possible reasons for recurrent respiratory tract infections, serum MBL was determined in 11 Colombian children

who had been suffering diseases such as pneumonia, otitis media and sinusitis, from which at least 50% were expected to be caused by pneumococci (Kertesz *et al.*, 1998). Children included in the study did not have any primary immunodeficiency or any other condition that explained their clinical problem IgM, IgG, IgA, C3, C4, IgG1, IgG2, IgG3 and IgG4 were within the normal range expected for their age (Table 3.1).

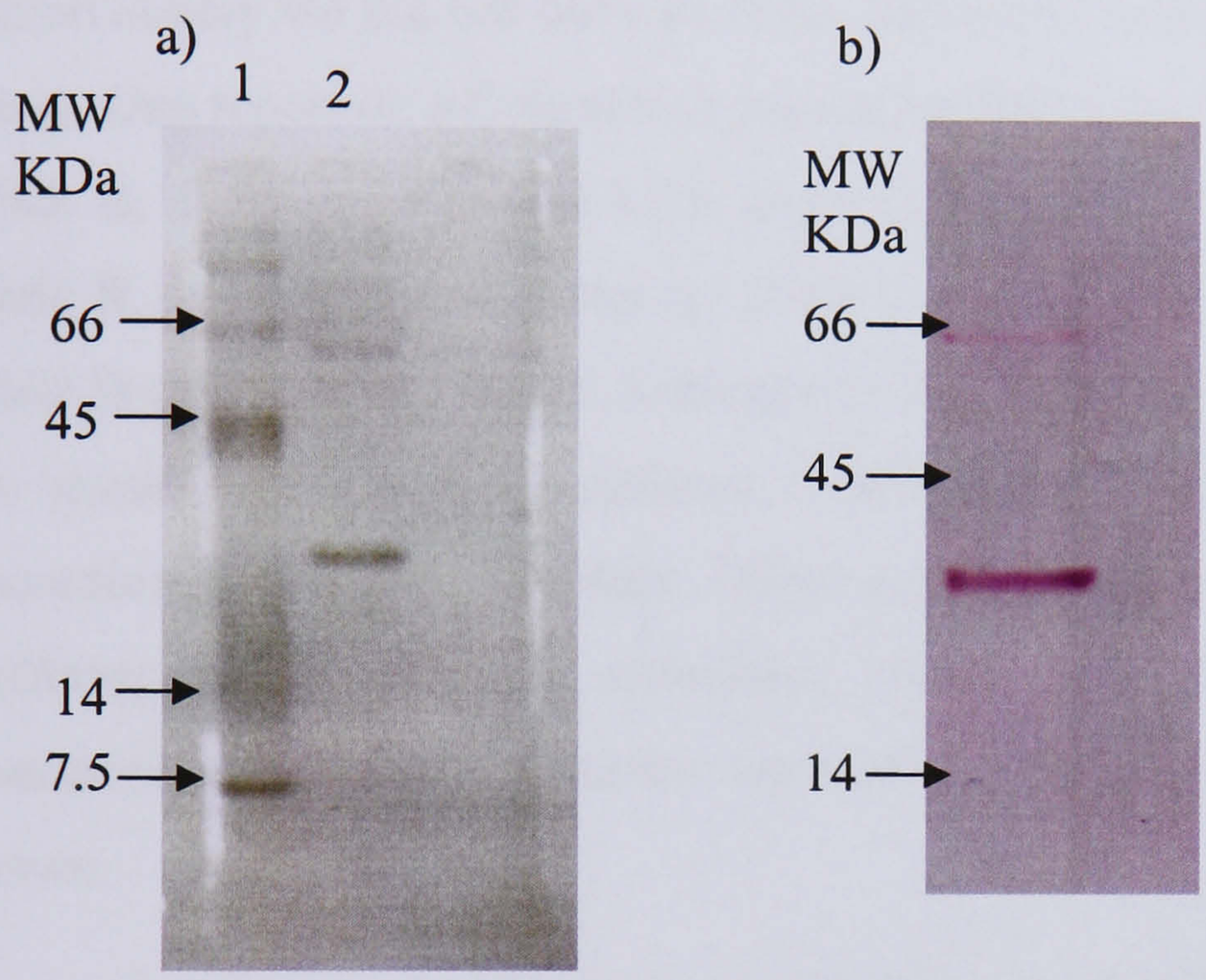


Figure 3.6. Purification of MBL. a) SDS PAGE of reduced purified human MBL stained with Coomassie blue. Lane 1 shows molecular markers and lane 2 MBL sample. b) immunoblot with a biotinylated mouse monoclonal anti-MBL antibody. In the immunoblot two bands can be seen one at approximately 29 kDa and another one at approximately 60 kDa, which reflects MBL plus MASP-2. This complex is difficult to separate (Methods section 2.1.2).

Concentrations of IgG antibody to *Streptococcus pneumoniae* serotypes 1, 3, 4, 5, 6B, 9V, 14, 18C, 19F and 23F were also determined. Antibody concentrations after vaccination with the 23-valent pneumococcal vaccine were considered normal when either a four-fold increase was achieved or when titers reached 1.3μg/ml for five or more pneumococcal serotypes (Sorensen *et al.*, 1998). In Figure 3.7 it can be seen that mean responses satisfied these criteria. Individually no child failed to respond to the pneumococcal vaccine.

Different pneumococcal serotypes have been grouped according to the response obtained. 1, 3, 4, 6B, 9V had lower levels than serotypes 5, 14, 18C, 19F and 23F, which is probably due to previous pneumococcal infection with these particular serotypes since they had higher pre-existing IgG antibodies. None of them, however, had MBL deficiency, which would be characterised by values below 1 μg/ml. (Figure 3.8).

Unfortunately we did not have an MBL deficiency serum to use as a control for this assay. One report on a Colombian population found 21.4% of 140 individuals having allele B, 3.6% allele D and 3.2% allele C. Five individuals were homozygous for allele B, one was homozygous for allele C and no individuals were homozygous for allele D (Malik *et al.*, 2003). Although no serum MBL concentrations were reported, we would expect 3 of the children in this study to have low MBL concentrations according to this genotype data. However, the group of children studied here were suffering from recurrent infections which may also increase serum MBL concentrations and the numbers were too small to permit any conclusions to be drawn.

Table 3.1. Serum concentrations of different immunoglobulin and complement components in selected Colombian children. Different immunological parameters were determined by nephelometry in 11 children. Data shows mean and range for each parameter.

Parameter	Mean (mg/dL)	Range (mg/dL)
IgM	99	41.2 - 229.6
IgG	1147.59	414 – 1614
IgA	109.2	37.5 - 272.9
C3	126.9	33.5 - 259.3
C4	34.5	21.7 - 62.5
IgG1	869.8	376.5 – 1080
IgG2	199.9	6.2 - 513.4
IgG3	44.3	13.6 – 166.1
IgG4	52.4	10 – 348.1

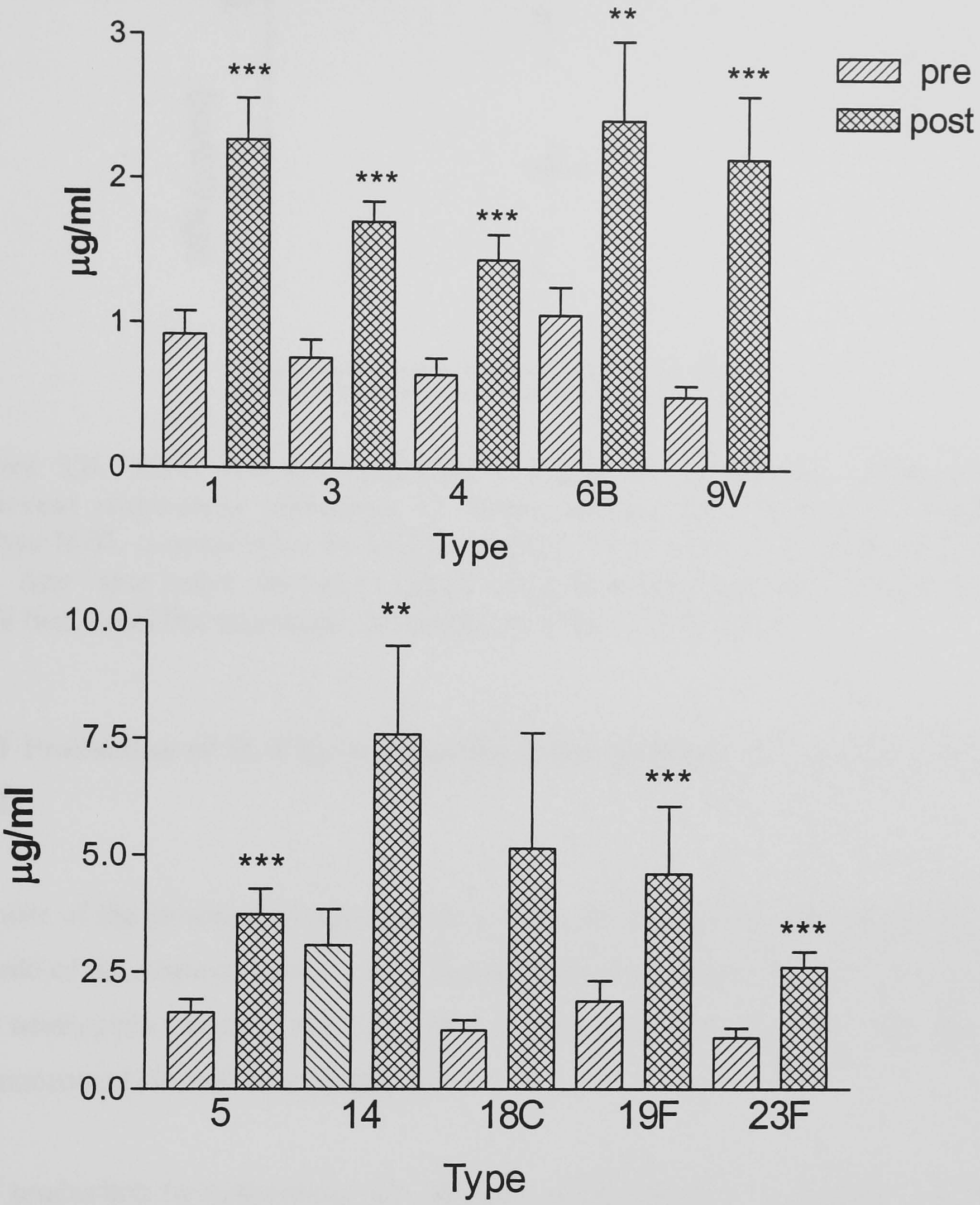


Figure 3.7. Specific IgG antibodies to different pneumococcal types in a group of Colombian children with recurrent infections. IgG specific anti- pneumococcal serotypes 1, 3, 4, 5, 6B, 9V, 14, 18C, 19F and 23F were determined by ELISA before and 4 weeks after vaccination with the 23-valent pneumococcal vaccine. 11 children suffering from recurrent pneumococcal infections have been analysed. Bars show mean and s.e.m and p values between titers pre and post vaccination obtained by Mann Whitney test. ** $p<0.01$, *** $p<0.001$

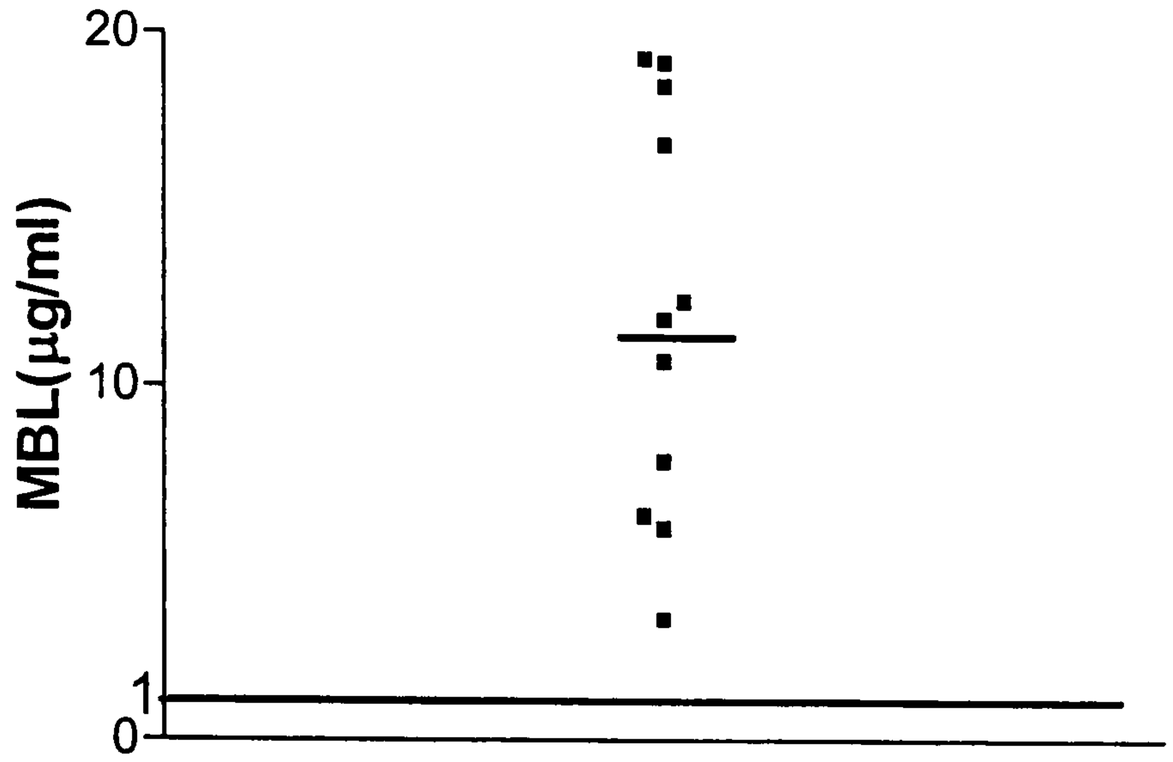


Figure 3.8. MBL concentrations in a group of Colombian children with recurrent respiratory infections. 11 serum samples from children were taken to analyse MBL concentration by ELISA (section 2.1.3) (each value represented by a dot). Any value below the line (1µg/ml) would be considered MBL deficiency. The small horizontal line represents the median of MBL (11.91 µg/ml).

3.4.3 Production of IL-8 by neutrophils in the presence of *S. pneumoniae* and MBL

Because of the presence of sugars such as N-acetyl glucosamine and mannose in the capsule of the pneumococcus it was thought that MBL might bind to them, interact with neutrophils through binding to CR1, a reported receptor for MBL, and facilitate pneumococcal clearance by phagocytes.

IL-8 production by neutrophils was assessed as an indicator of possible interaction with MBL when in the presence of encapsulated (type 3) or non-encapsulated (R36A) *Streptococcus pneumoniae*. IL-8 synthesis was compared with the one induced by a recognised organism (*Cryptococcus neoformans*) able to bind to MBL and therefore with the capacity to interact with CR1 expressed on neutrophils.

In Table 3.2 it can be observed that *Cryptococcus neoformans* alone induced IL-8 production in neutrophils. The production of the chemokine increased by a factor of two when the organism was incubated with MBL at 10µg/ml. On the contrary, both encapsulated and non-encapsulated *Streptococcus pneumoniae* were not able to induce more IL-8 in the presence of the same concentration of MBL.

Stimulus	None (n=2)	*S. pneumoniae* type 3 (n=2)	*S. pneumoniae* R36A (n=2)	*Cryptococcus neoformans*
	IL-8 (pg/ml)			
None	123, 72.5	373.06, 384.5	373.26, 385.8	750
LPS	2200, 1200	3200, 1345	1800, 1560	4200
MBL	105.6, 86.97	332.4, 325.8	389.5, 377.8	1550

Table 3.2. Effect of MBL on IL-8 synthesis by neutrophils. Neutrophils (2 x 10^6/ml), isolated by dextran, were cultured overnight in the presence or absence of the following stimuli: *S. pneumoniae* type 3 or R36A at a ratio 1:30 (neutrophils: pneumococcus), LPS 1 μg/ml, MBL at 10 μg/ml or *C. neoformans*. IL-8 production (pg/ml) in the supernatants was determined by ELISA. Values show data from 2 experiments on two different donors, except for *C. neoformans* where the values from 1 experiment are shown.

Proteases from the pneumococcus such as pspA or IgA1 protease may affect these results. A known quantity of IL-8 was incubated with different concentrations of pneumococcus, similar to the ones used in the study, to see whether, after overnight incubation incubation, levels of IL-8 were altered. We found that after overnight incubation similar concentrations of IL-8 were measured (Figure 3.9), a finding that rejects the possible effect of pneumococcal proteases

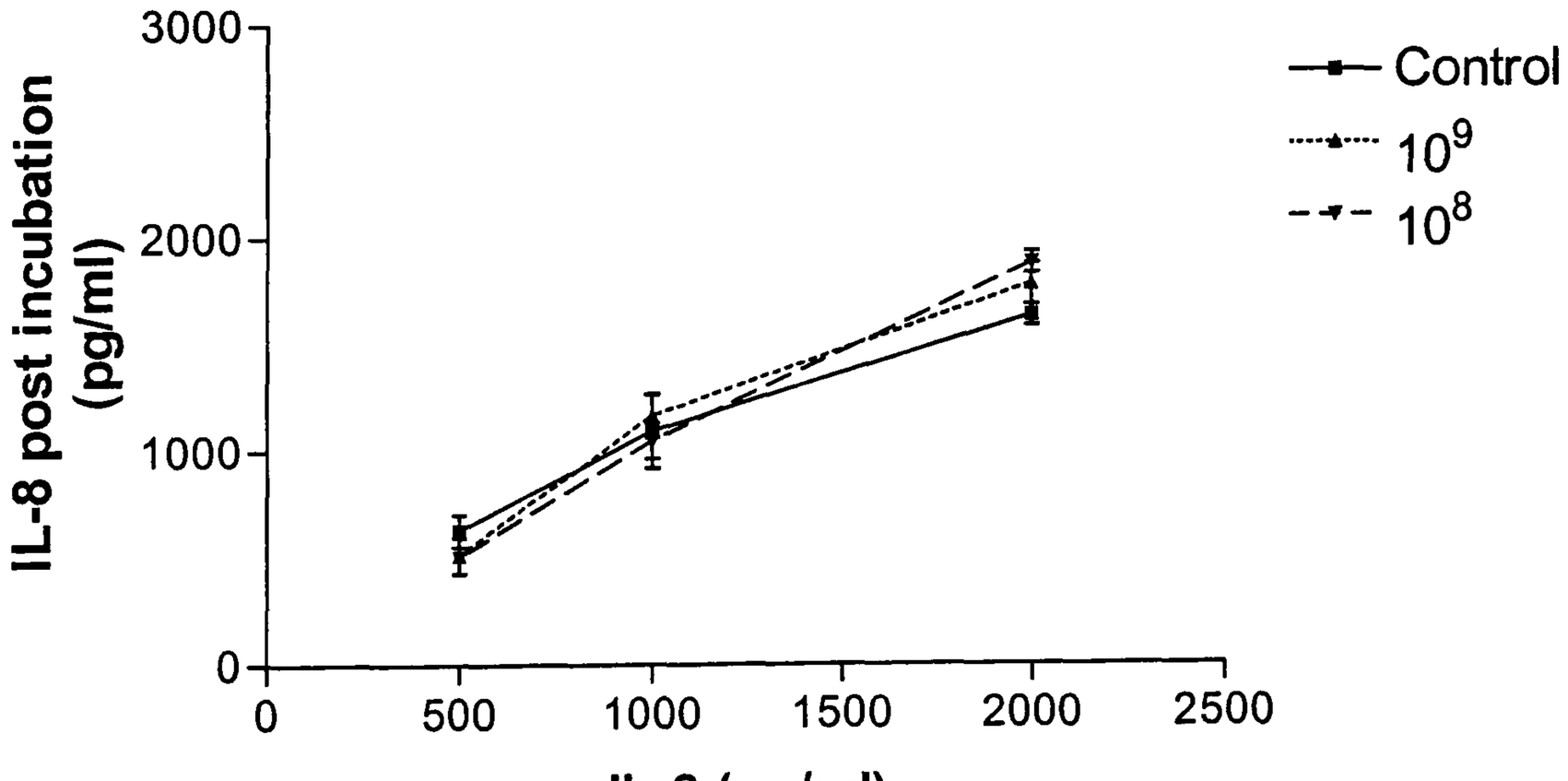


Figure 3.9. *Streptococcus pneumoniae* does not alter IL-8 concentration. Different numbers of bacteria (0, 10^8 and 10^9/ml) were cultured overnight in the presence of IL-8 at 500, 1000 and 2000 pg/ml, next day, following centrifugation, the supernatants were assayed for IL-8 by ELISA.

3.4.4 MBL does not bind to *S. pneumoniae* serotype 3.

As mentioned before the presence of sugars on the capsule of the pneumococcus suggested a possible interaction with MBL. We had the aim of comparing MBL with CRP when investigating the effect of innate opsonins on neutrophil/ *S. pneumoniae* interactions. For this reason, binding of MBL to *S. pneumoniae* was examined by FACS using a FITC-labelled antibody to MBL and it was compared to the binding to *C. neoformans* which was previously shown to bind to MBL (Schelenz *et al.*, 1995) (Figure 3.10). It is clear that MBL is able to bind to *C. neoformans* (panel b), whereas no binding can be observed to the pneumococcus (panel a). This result agrees with recent reports arguing against the capacity of MBL to bind pneumococci (Neth *et al.*, 2000).

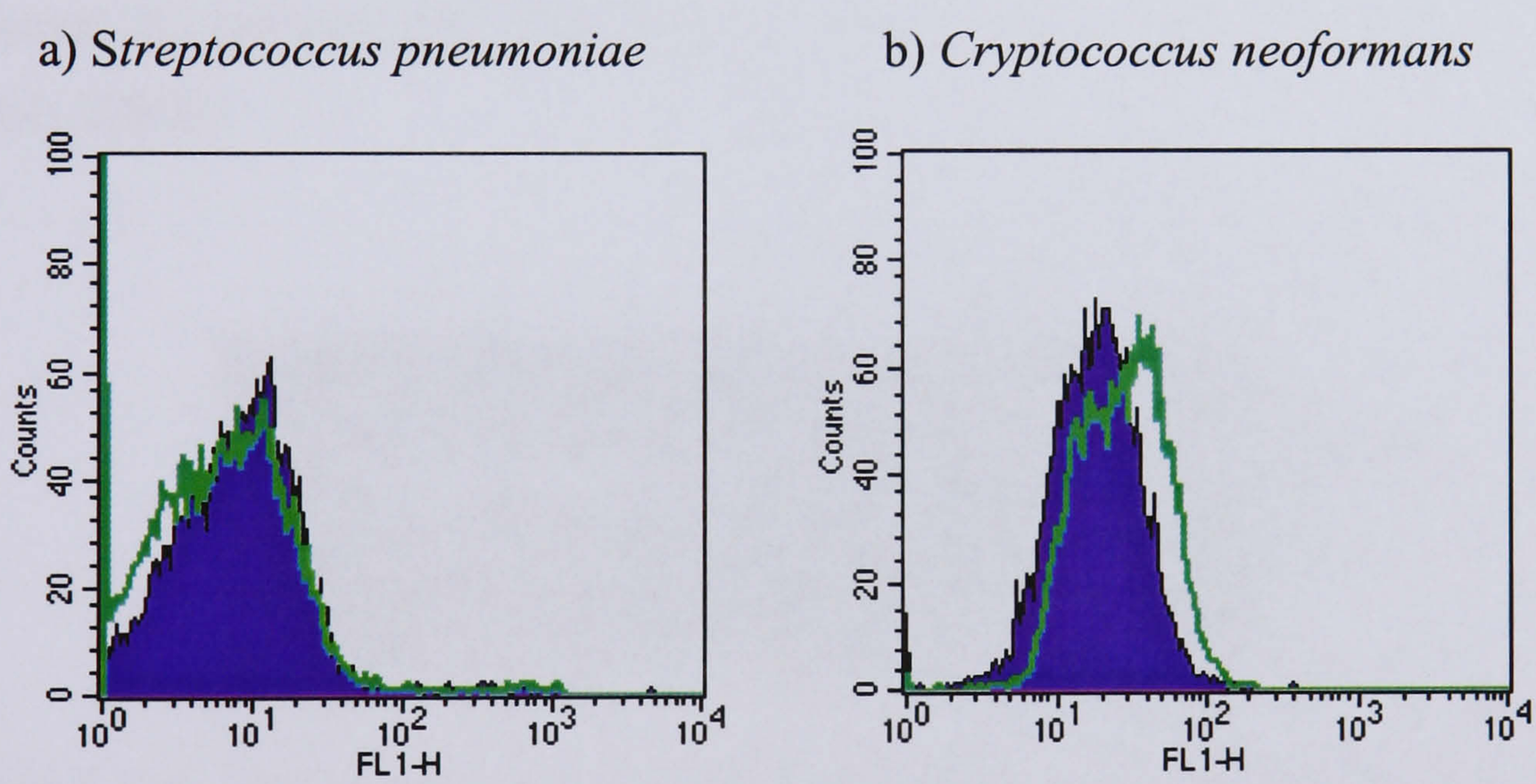


Figure 3.10. MBL binds to *Cryptococcus neoformans* but not to *Streptococcus pneumoniae*. 10 μg/ml of MBL were incubated with 5 x10^6 of each micro-organism in the presence of 10 mM calcium in HBSS during 1 hour at 4°C. An IgG1 mouse biotinylated antibody anti-MBL was added and streptavidin- FITC conjugated was used to detect antibody binding —. An IgG1 mouse isotype control ■ was used for comparison. At least 30000 events were acquired to analyse binding. Graph shows a representative experiment from two that gave similar results

3.5 Effects of CRP on neutrophil function

In order to determine the capacity of CRP to alter neutrophil function we chose to measure IL-8 and NADPH oxidase activity as markers of neutrophil activation and were measured by ELISA and DHR-123 oxidation by FACS respectively. The effect of different concentrations of CRP in the presence or absence of pneumococci were analysed and compared.

3.5.1 Polymorphisms of FcγRIIA

CRP has been shown to bind to both FcγRI and may also bind to FcγRIIA and both are expressed on neutrophils. However, binding to FcγRIIA has been reported to be affected by polymorphisms at position 131 on the second external loop of the molecule. FcγRIIA with arginine in that position (RR-homozygous) binds CRP whereas the histidine (HH homozygous) form shows very weak or no binding (Stein *et al.*, 2000b).

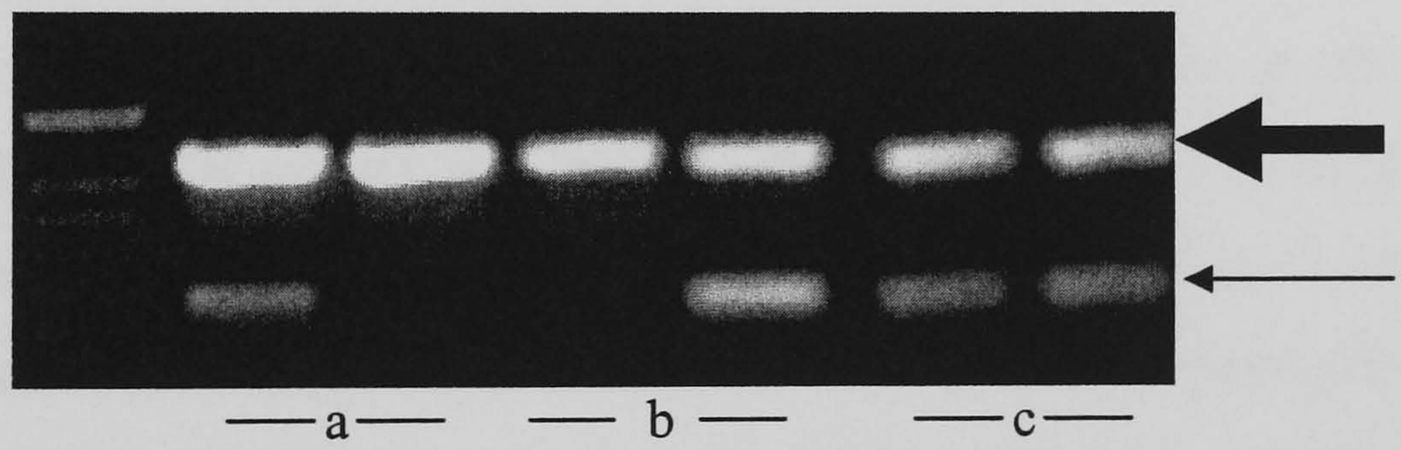


Figure 3.11. Polymorphisms of FcγRIIA (Section 2.4.2). The bigger arrow shows the amplification of human growth hormone (439bp) as an internal control, whereas the smaller arrow shows amplification of FcγRIIA (253bp).Lanes 1, 3, 5 show amplification with H131 specific primer and 2,4,6 show amplification with R131 specific primer. Tracks 1 and 2: DNA from donor a; 3 and 4 donor b and 5 and 6 donor c. The gel shows that a is homozygous H; b is homozygous R and c is heterozygous

The FcγRIIA polymorphisms were determined in 34 adult healthy volunteers. Of these 16 were heterozygous because they showed bands for both forms (as lanes 5 and 6 in Figure 3.11), whereas 9 were homozygous for R131 and 9 were homozygous for H131. This data follows the Hardy-Weinberg equilibrium.

As an example of what it is seen after agarose separation, three different donors are shown in Figure 3.11, where donor a (lanes 1 and 2) is homozygous for H131, donor b (lanes 3 and 4) is homozygous for R131 and donor c (lanes 5 and 6) is heterozygous.

3.5.2 Induction of IL-8 production by neutrophils following interaction with CRP and *S. pneumoniae*

IL-8 is one of the major chemokines synthesised by neutrophils in an autocrine way to attract cells to the site where an inflammatory process is starting. We assessed the ability of CRP at various concentrations to induce production of IL-8 by neutrophils and also looked for differences in the IL-8 produced by neutrophils from HH and RR homozygous donors.

Increasing concentrations of CRP on its own were able to induce the synthesis of IL-8 in both types of neutrophil. No difference was observed in the level of IL-8 produced by neutrophils from HH donors in comparison to RR donors as shown in Figure 3.12. Moreover, no difference was observed when IFNγ at 100 IU/ml was added as a stimulus. *S. pneumoniae* serotype 3 induced a higher IL-8 response than that seen in its absence and again was lower in RR donors with and without CRP at various concentrations (Figure 3.12 panels b and d). Increasing concentrations of IL-8 in the presence of CRP was seen using R36A, which is a non-encapsulated pneumococcus (Figure 3.13), although the organism on its own induced less IL-8 than the encapsulated type 3.

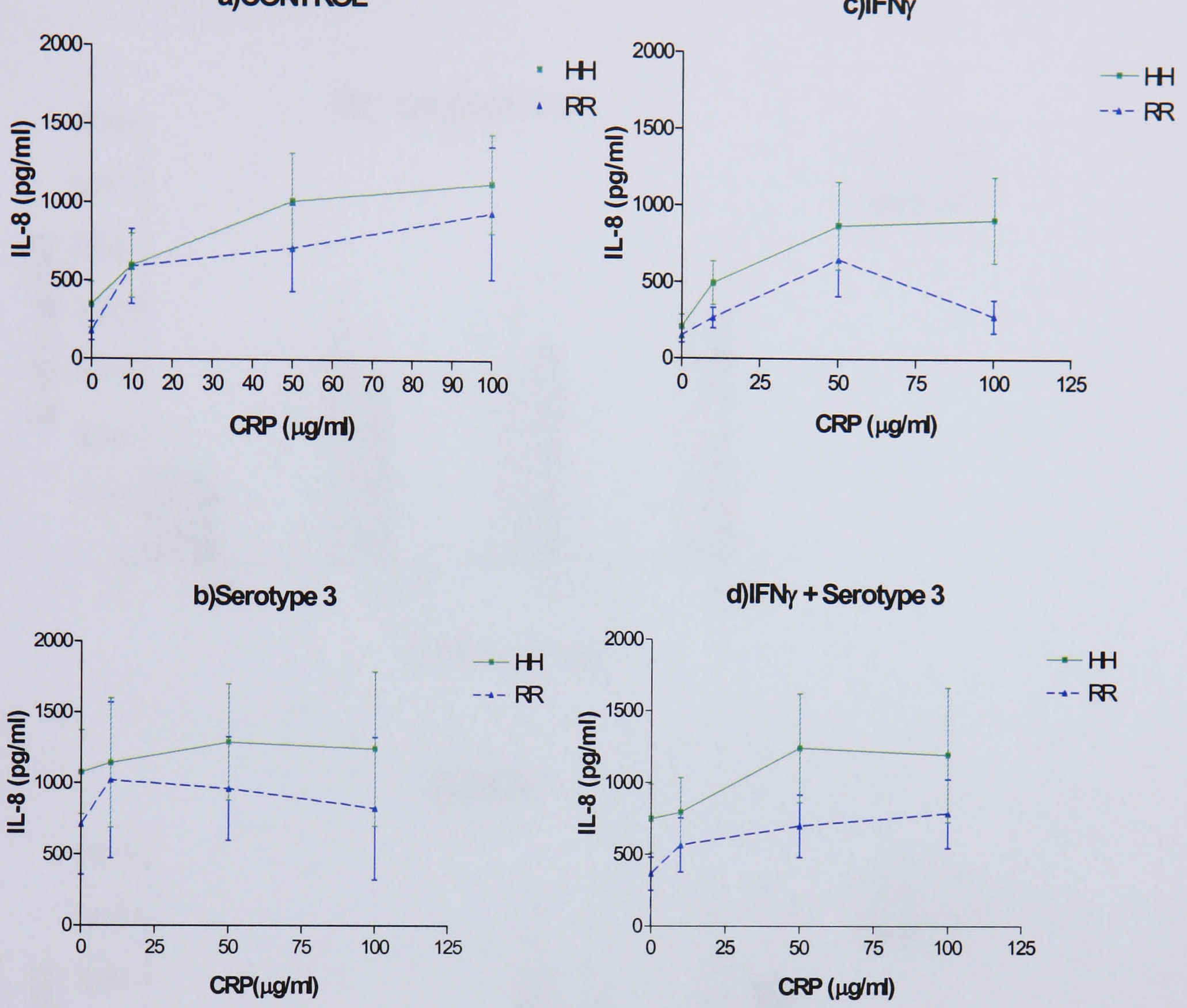


Figure 3.12. Effect of CRP and *S. pneumoniae* on IL-8 production by isolated neutrophils. Isolated neutrophils from both HH and RR homozygous individuals were cultured overnight at 37ºC in the presence of *S. pneumoniae* type 3 and CRP at 0, 10, 50 and 100 µg/ml. In some tests IFNγ at 100 IU/ml was added (c and d) . Supernatants were obtained, and IL-8 production (pg/ml) was determined by ELISA. Graphs show means and s.e.m of IL-8 produced by 6 different donors for each polymorphism. No significance statistical difference was found between responses obtained on neutrophils from HH and RR donors for this set of experiments when analysed by Mann Whitney U test.

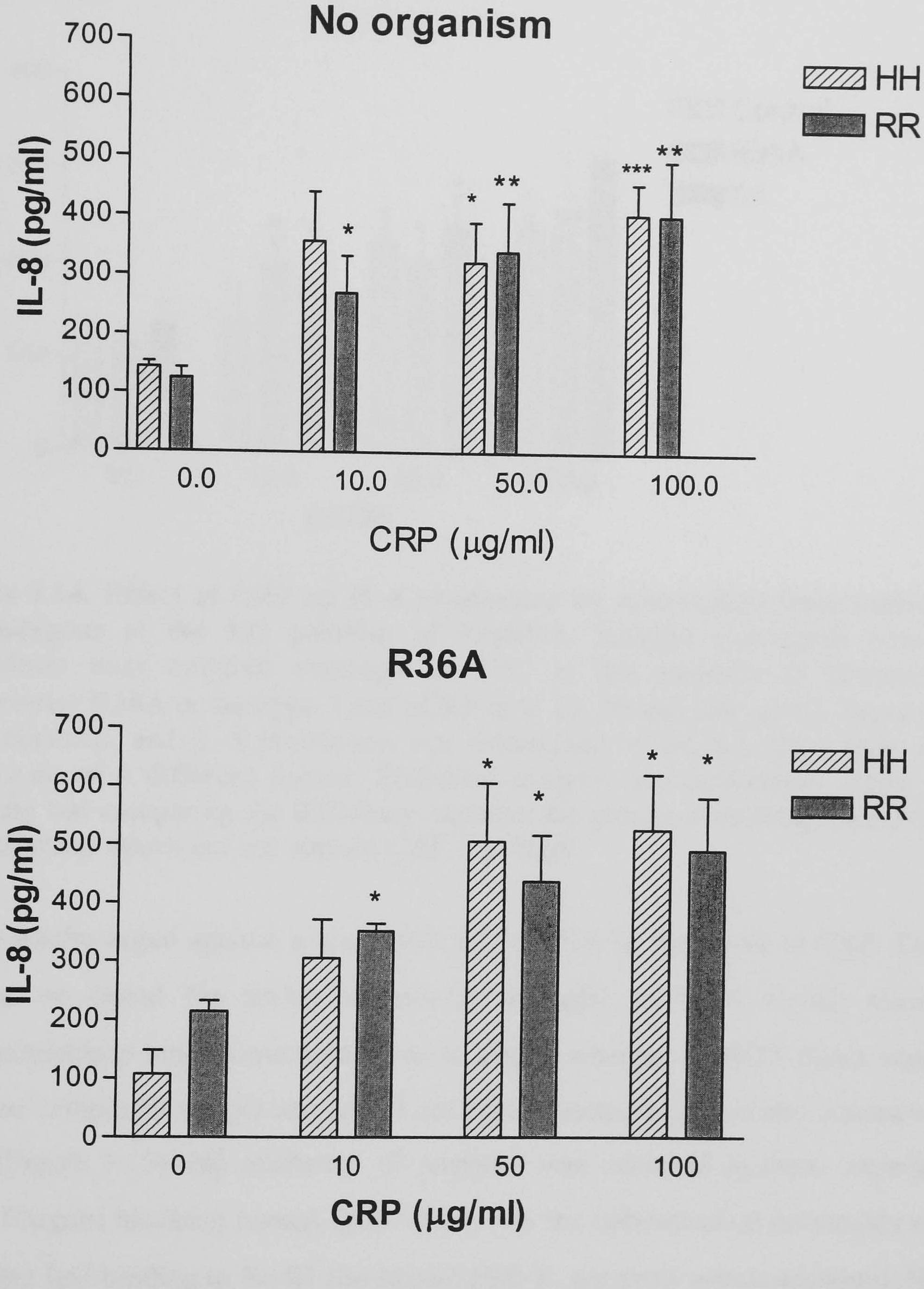


Figure 3.13 Effect of CRP and non-encapsulated *S. pneumoniae* on IL-8 production by neutrophils. Isolated neutrophils from individuals homozygous HH or RR at the 131 position of FcγRIIA were cultured overnight at 37°C in the presence of *Streptococcus pneumoniae* R36A and CRP at 0, 10, 50 and 100 µg/ml. Supernatants were obtained, and IL-8 production (pg/ml) was determined by ELISA. Graphs show means and s.e.m of 6 different HH donors and 6 RR donors. Statistical analysis was performed using the Mann Whitney test comparing presence of CRP with the control group that did not contain CRP. * $p<0.05$; ** $p< 0.01$; *** $p<0.001$

Experiments performed with heterozygous donors gave a similar result, in that CRP at increasing concentrations was also able to induce synthesis of IL-8 by neutrophils (Figure 3.14).

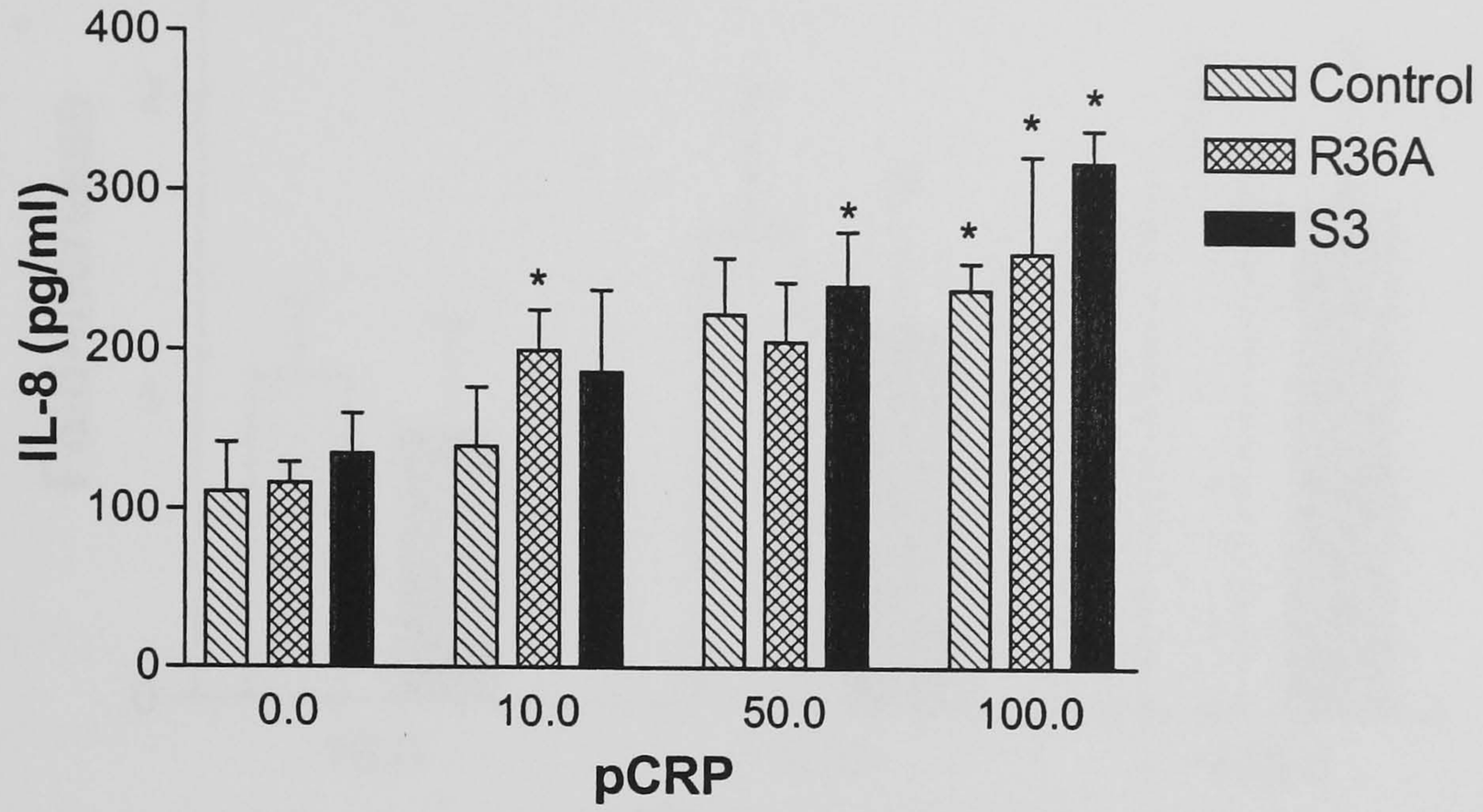


Figure 3.14. Effect of CRP on IL-8 production by neutrophils from individuals heterozygous at the 131 position of FcγRIIA. Isolated neutrophils from such individuals were cultured overnight at 37°C in the presence of *Streptococcus pneumoniae* R36A or serotype 3 and pCRP at 0, 10, 50 and 100 μg/ml. Supernatants were obtained, and IL-8 production was determined by ELISA. Bars show means and s.e.m. of 4 different donors. Statistical analysis was performed using Mann Whitney test comparing the difference between the groups containing CRP with the control group which did not contain CRP. * $p<0.05$

These results argue against a major role of FcγRIIA in responses to CRP. For that reason, we tested the ability of monomeric IgG1 to block FcγRI, since this immunoglobulin binds particularly well to FcγRI, whereas FcγRIIA binds mostly to immune complexes of IgG and would not bind monomeric IgG at the concentrations used (Figure 3.15). No inhibition of response was achieved in these experiments using 10μg/ml blocking human IgG1 which was the concentration previously shown to inhibit IgG binding to FcγRI (Bodman-Smith K. personal communication). Higher concentrations of IgG should be tested to see if clearer inhibition is obtained. In HH donors we observed a tendency to produce more IL-8, but it was not statistically significant.

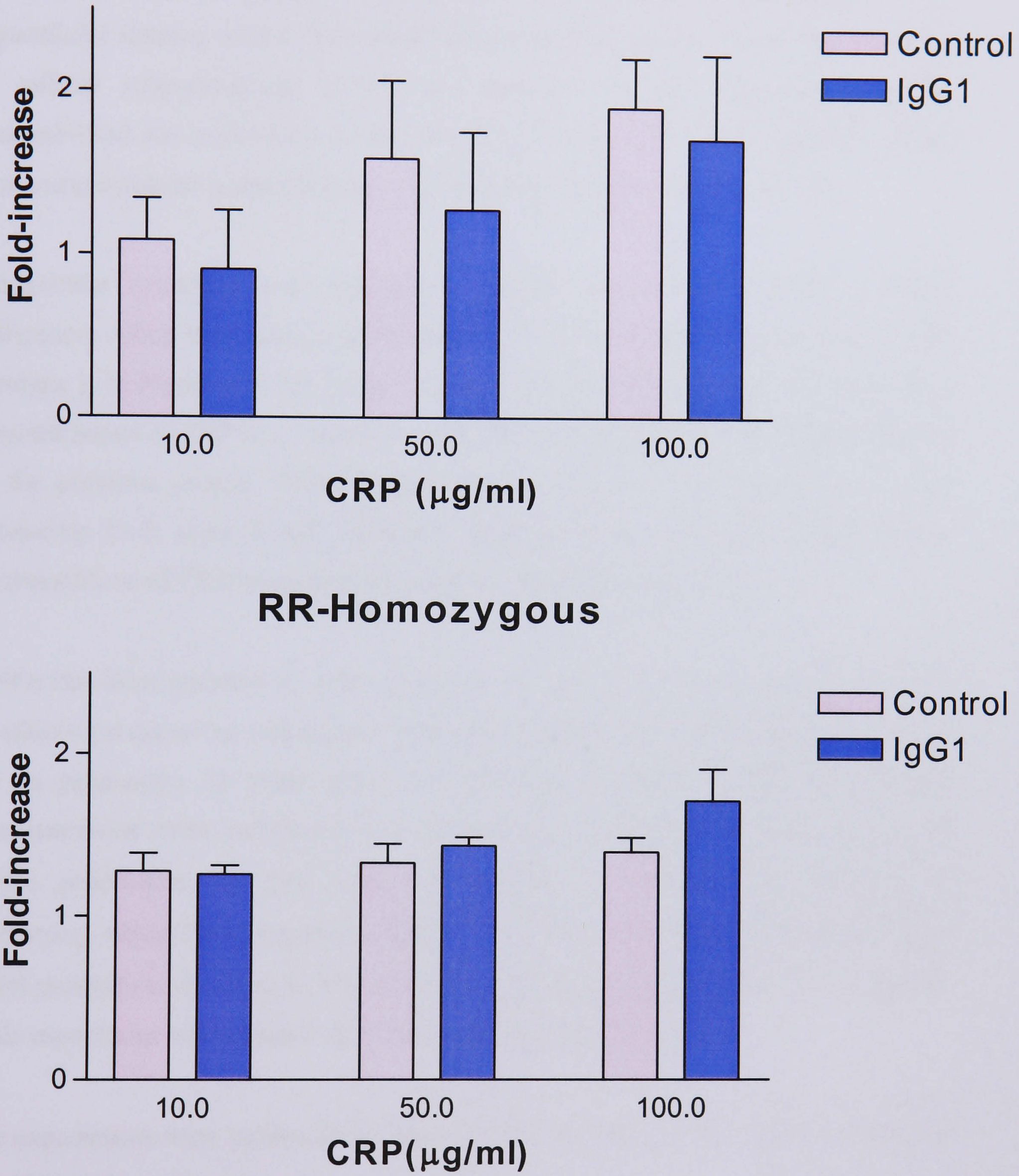


Figure 3.15 Effect of blocking FcγRI on IL-8 production by neutrophils. Neutrophils from HH and RR donors were incubated with IFNγ at 100 IU/ml overnight at 37°C. Next day, monomeric IgG1 at 10 μg/ml was incubated with cells for 30 minutes at 4°C. Fold-increase in the production of IL-8 compared to that in the absence of CRP was calculated. Bars representing means and s.e.m. from four different donors of each allotype are shown.

3.5.3 Intracellular cytokine production by neutrophils

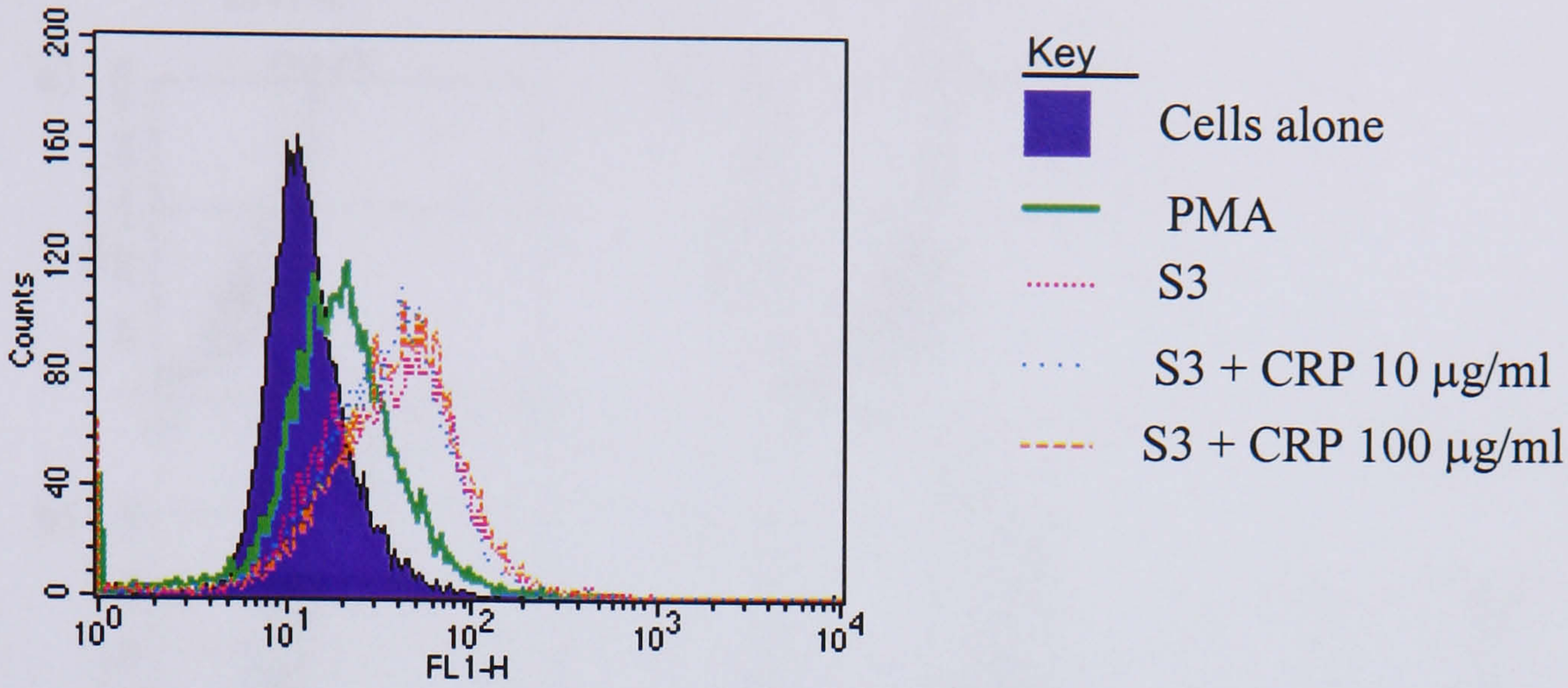
A different way to assess the production of cytokines by neutrophils is by intracellular staining with a fluorescent labelled antibody which allows the definition of cellular subpopulations if they are present. In this approach cells are permeabilised and brefeldin A is used to prevent secretion of these molecules. Thus, measuring cytokines intracellularly may have higher sensitivity than ELISA.

Intracellular cytokines were analysed in isolated neutrophils in order to define differences when increasing concentrations of CRP were used in the presence of serotype 3. In Figure 3.16 the production of IL-8 was slightly higher when increasing concentrations of CRP were added on its own, in accordance with the data presented in the previous section. When pneumococcus serotype 3 was added, more cells producing IL-8 were found. However, there was no difference when various concentrations of CRP were added to *S. pneumoniae* and neutrophils.

TNFα has been reported by some groups to be one of the major pro-inflammatory cytokines produced by neutrophils. However, analysis by ELISA demonstrated low or no production by these cells. The presence of MBL or CRP at different concentrations even combined with *Streptococcus pneumoniae* failed to induce TNFα production. For this reason, we decided to determine this cytokine by measuring intracellular synthesis. Figure 3.17 shows that even at the intracellular level production of TNFα is low or non existent when compared to IL-8 synthesis. This experiment was repeated three times with the same results.

No experiments were performed by first priming neutrophils with IFNγ or GM-CSF to elicit TNFα production.

a)



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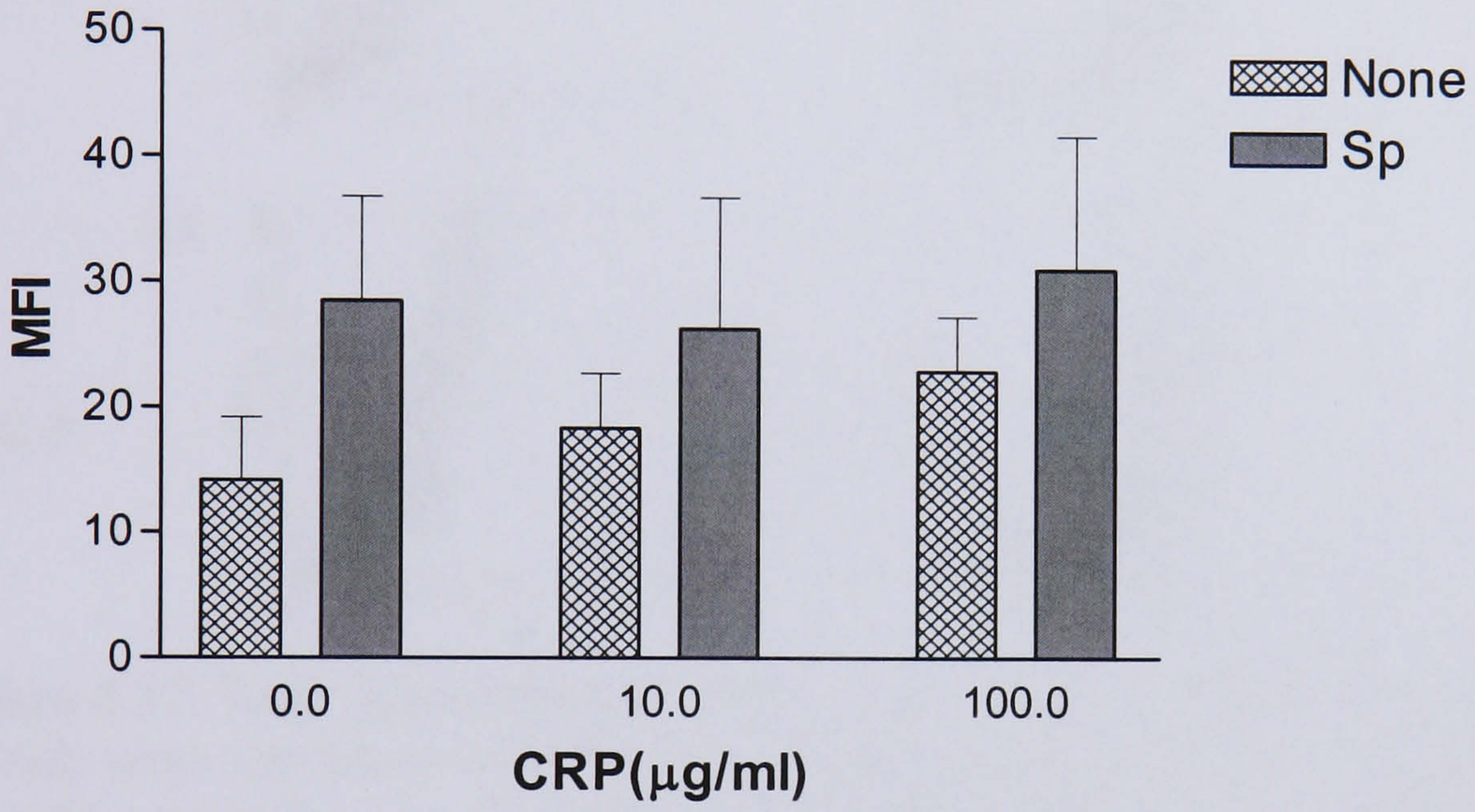


Figure 3.16. Intracellular IL-8 production by isolated neutrophils. Neutrophils at 5 x 10^6/ml were incubated with or without *S. pneumoniae* type 3 at a ratio 30:1 and different concentrations of CRP: 0, 10 and 100 µg/ml. In panel a the histogram shows the effect of CRP after 30000 events were acquired, whereas in panel b the means ± s.e.m. fluorescence intensity values of 3 different experiments are represented. No statistically significant differences were obtained when data were analysed by Mann Whitney U test.

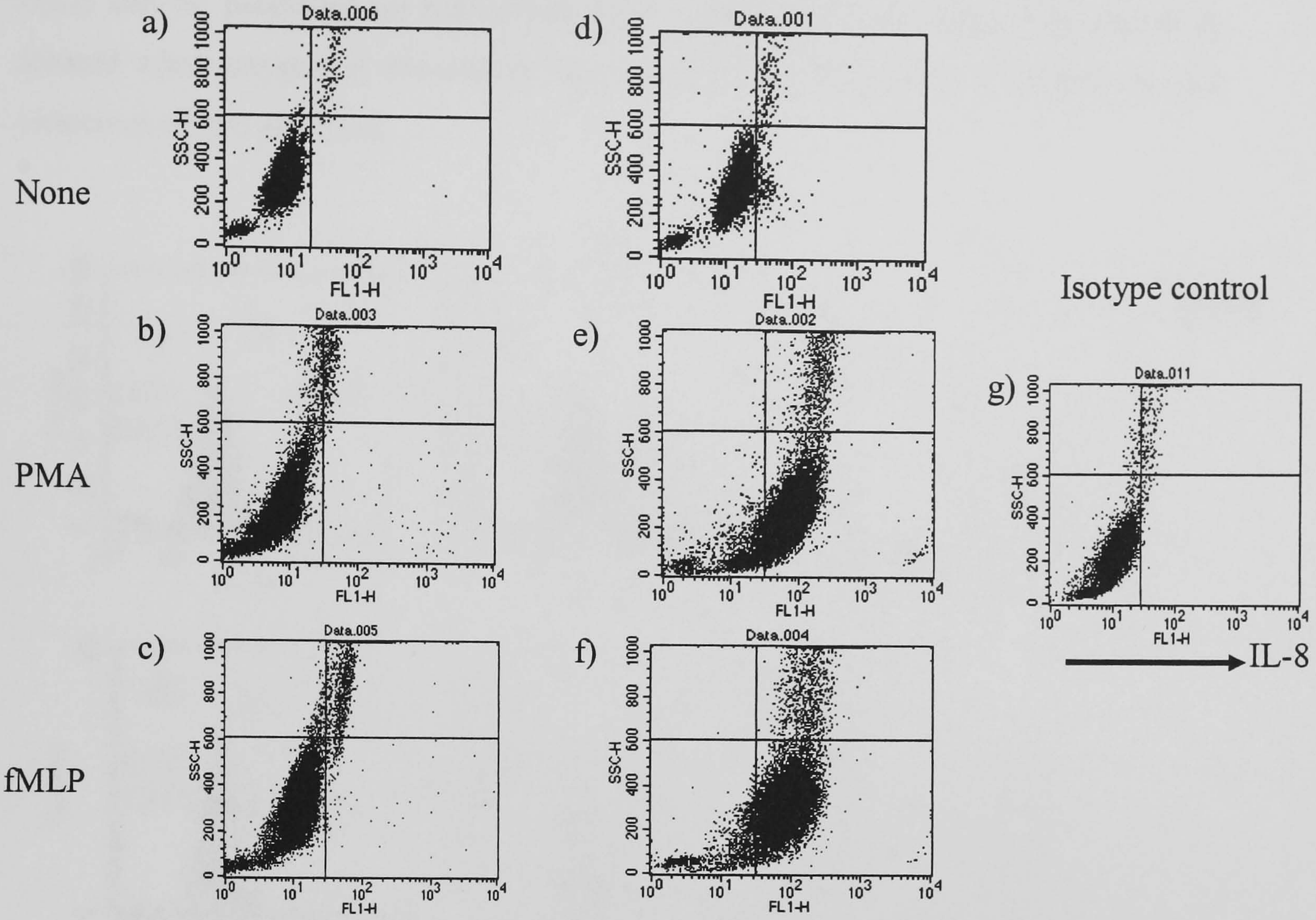


Figure 3.17. Low production of TNFα by isolated neutrophils. Neutrophils (5 x 10^6/ml) were incubated with different conditions: no stimulus, panels a and d; PMA at 5 μM, panels b and e; and fMLP at 100nM in panels c and f. TNFα synthesis is shown in panels a, b and c whereas IL-8 production can be seen in panels d, e and f. A mouse IgG1 antibody to TNFα and another to IL-8 was used according to the conditions described in section 2.7.2.3; an isotype control for these antibodies can be observed in panel g. The figure shows a typical result from 3 different experiments.

3.5.4 NADPH oxidase activity of isolated neutrophils

FcγRIIA aggregation has been reported to cause reactive oxygen synthesis. Therefore we tested the ability of CRP to activate neutrophils. Neutrophils are professional phagocytes, and one way to kill micro-organisms is by synthesis of highly toxic oxidative radicals derived from oxygen, which are produced by the action of NADPH oxidase. After the activation of neutrophils, cytosolic proteins translocate to a membrane bound cytochrome b, which helps to catalyse the reduction of molecular oxygen at the expense of NADPH. Since the cytochrome b can be present in the plasma membrane or in the membrane of intracellular granules, oxygen can be reduced inside or outside the cell.

Depending on the number of electrons by which oxygen is reduced superoxide or H_2O_2 can be produced on both sides of the neutrophil membrane. Superoxide is formed when oxygen is reduced by one electron, whereas H_2O_2 is formed by the reduction of two electrons.

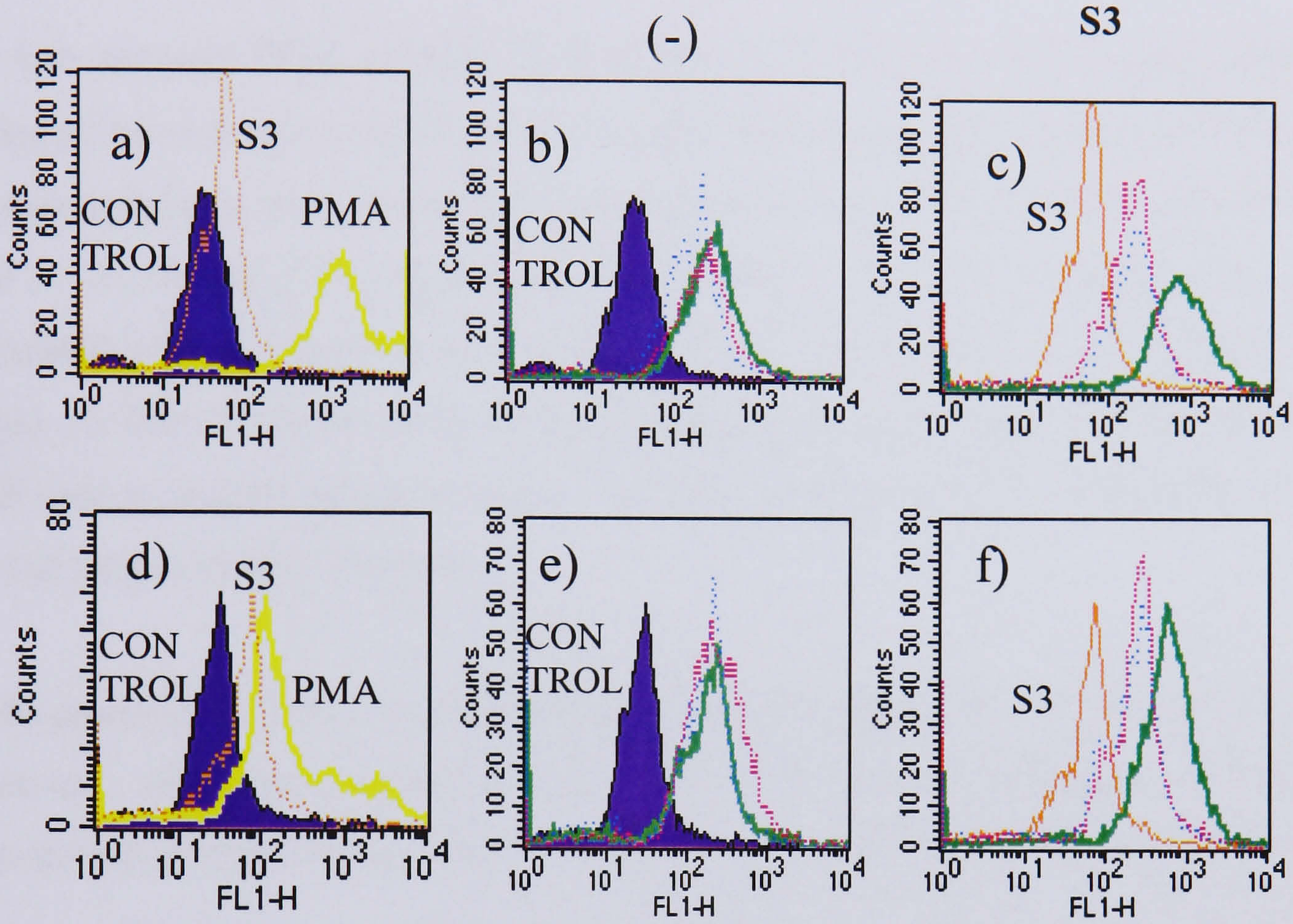


Figure 3.18. Effect of different concentrations of CRP on the oxidation of DHR 123 by neutrophils from an HH-homozygous donor. Isolated neutrophils at 2 x 10^6 cells/ml have been incubated overnight with 100 IU/ml IFNγ (d,e,f) or without IFNγ (a,b,c). Cells were untreated (blue filled hstogram) treated with PMA (yellow line) or *S. pneumoniae* (dotted orange line) followed by DHR 123 (a,d). Cells were also treated with *S. pneumoniae* serotype 3 at a ratio 30:1 together with CRP at 10, 50 or 100 μg/ml (c,f) or with CRP in the absence of bacteria followed by DHR 123. A down-regulatory effect is seen at increasing concentrations of CRP; CRP 10 μg/ml (green histogram) CRP 50 μg/ml (dotted red histogram) and CRP100 μg/ml (dotted blue histogram).

Oxidation of dihydrorhodamine 123 (DHR) to rhodamine is one useful way to determine intracellular H_2O_2 and this fluorescence can be analysed by FACS. Once rhodamine is formed it cannot diffuse out of the cell to the extracellular medium, which makes it a reliable method for assessment of intracellular H_2O_2 by different stimuli (Henderson and Chappell, 1993). For this reason, this technique was used to determine the effects of various concentrations of CRP in the presence or absence of

S. pneumoniae serotype 3 in different donors. One illustrative experiment is shown in Figure 3.18 where CRP at low concentrations initially increases the response but increasing concentrations of CRP appear to down-regulate production of intracellular H_2O_2 independently of the presence of IFNγ.

We then analysed DHR oxidation in both HH- and RR-donors to see if the presence of this polymorphism in the FcγRIIA molecule confers any difference to the NADPH oxidase activity in the presence of various concentrations of CRP and *S. pneumoniae* type 3. In Figure 3.19, where fold increments in the MFI of DHR have been calculated over the baseline, it is shown that the highest value was observed at 10 μg/ml of CRP in the presence of *S. pneumoniae* whereas higher concentrations of CRP tend to induce values similar to controls. This effect was independent of the type of polymorphism analysed.

In the presence of IFNγ a similar pattern of greatest response at 5-10 ug/ml CRP was observed. HH donors showed a tendency towards a higher production of radicals than did RR donors. This pattern was similar to that observed for IL-8 production.

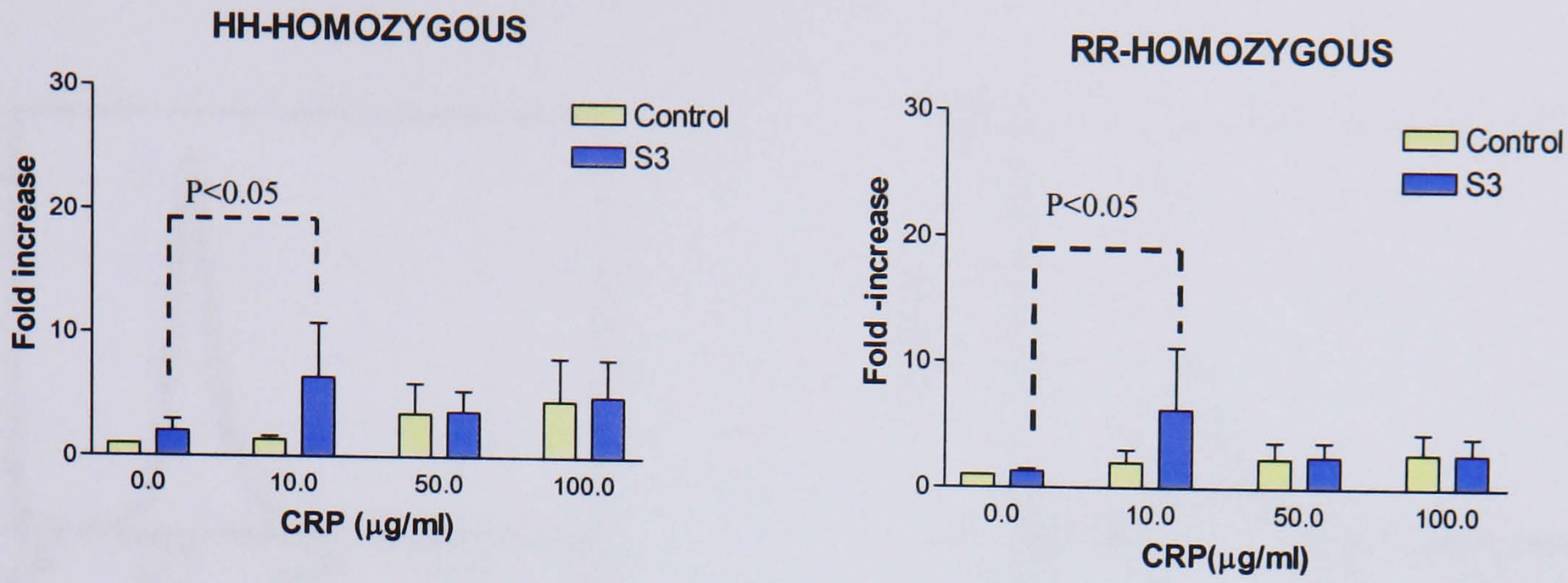


Figure 3.19. Oxidation of DHR123 by isolated neutrophils occurs maximally at 10 µg/ml CRP. Isolated neutrophils at 2 x 10^6/ml from HH and RR donors were incubated with or without IFNγ (100 IU/ml) and with or without *S. pneumoniae* at a ratio 30:1. CRP at 3 different concentrations was incubated with neutrophils for 30 minutes and oxidation of DHR 123 determined. The figure shows fold increases in the fluorescence obtained compared to fluorescence in the absence of CRP or *S. pneumoniae* serotype 3 for each donor. Means and s.e.m. for 4 different donors of each polymorphism are shown. Data were analysed using Mann Whitney test.

Because the activity of NADPH oxidase can also be affected by the priming action of some cytokines, neutrophils were incubated with different concentrations of CRP in the presence of IFNγ, IFNγ + TNFα or IFNγ + GM-CSF (Figure 3.20). Combining cytokines may be more similar to *in vivo* conditions when more than one cytokine are acting either synergistically or in an antagonistic way.

It was expected that IFNγ, TNFα and GM-CSF would work in a synergistic manner to stimulate neutrophils. In fact, using combinations of these cytokines the down-regulatory effect of CRP mentioned above in the absence of *S. pneumoniae* was more evident.

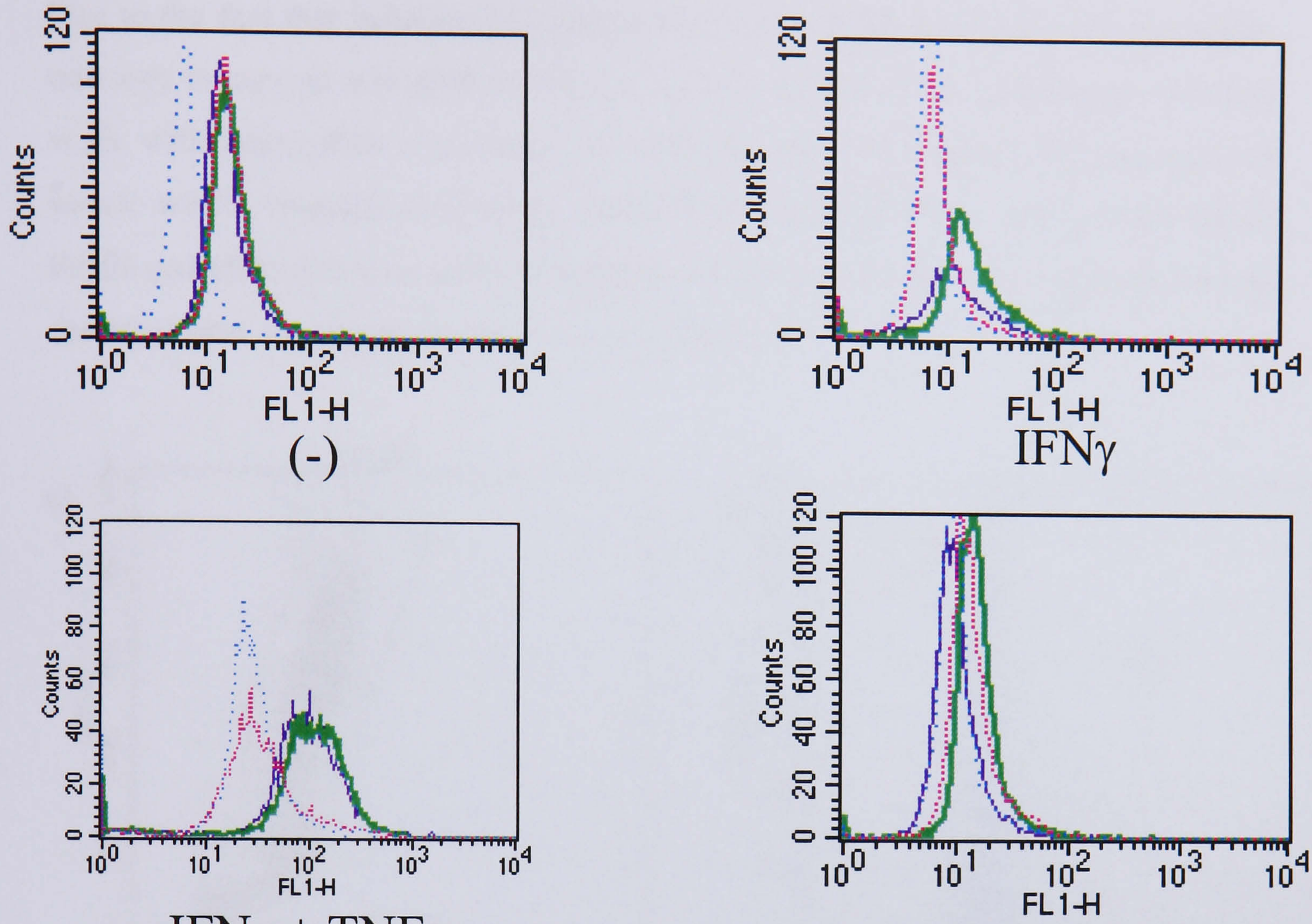


Figure 3.20. Effect of CRP on the oxidation of DHR 123 in primed neutrophils. 2 x 10^6/ml isolated neutrophils were cultured overnight with either TNFα (2 ng/ml), IFNγ (100 IU/ml) or GM-CSF (10 ng/ml) and the next day were treated with CRP at three different concentrations 0 ····· , 10 ——, 50 —— and 100 μg/ml ····· . At least 10000 events were acquired by FACS. Data for one representative result of two different experiments are shown.

The response observed is not dependent on the presence of pneumococci as CRP alone is able to reproduce it. However, the fact that a priming effect was observed suggests the presence of an inducible receptor for CRP on neutrophils. Since FcγRIIA is not inducible, and the fact that there was no correlation with polymorphisms in that receptor suggests that the effect was possibly due to interactions with FcγRI, the high affinity receptor, FcγRIIB or an unreported CRP receptor.

3.5.5 NADPH Oxidase activity in neutrophils maintained in whole blood

Due to the fact that isolation procedures may alter the baseline state of neutrophils, one way to prevent any artifact effect as a consequence of the techniques used is to work with neutrophils maintained in whole blood. Importantly, the presence of serum which contains circulating monomeric IgG, especially IgG1, could block FcγRI and affect the interaction of monomeric (pentamer) CRP with this receptor and therefore alter the responses observed in neutrophils.

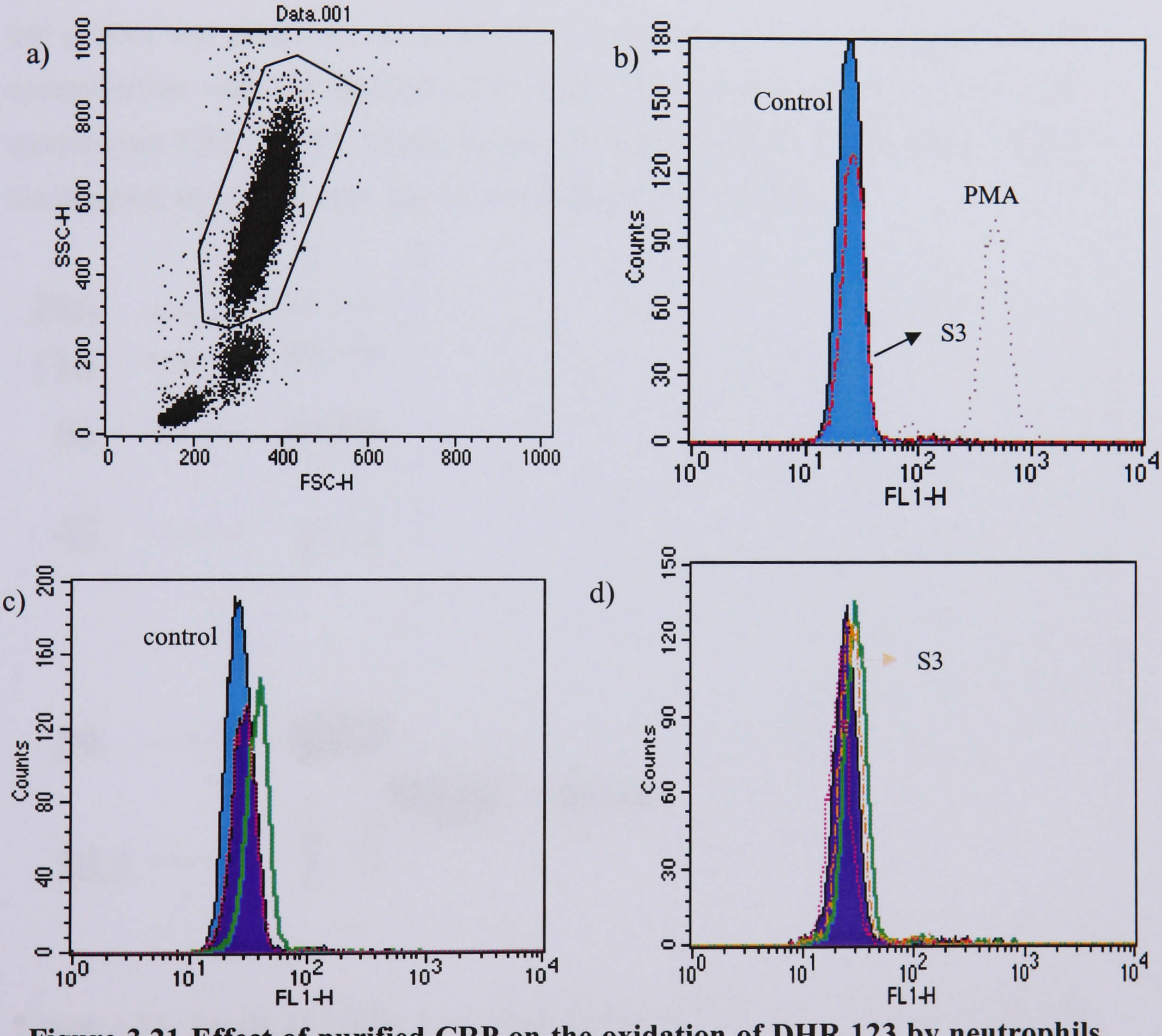


Figure 3.21 Effect of purified CRP on the oxidation of DHR 123 by neutrophils maintained in whole blood. Neutrophils have been gated as shown in panel a. Panel b shows a negative control (filled histogram) and a positive control with PMA at 5 μM (open histogram on the right). Panel c shows the response in the presence of CRP alone, whereas panel d shows the response in the presence of CRP and *S. pneumoniae* at 1 x 10^7 /ml. CRP at 10 ■ ,50 ▬ and 100 μg/ml ┅. These data are representative of four different experiments.

However, CRP in the absence or presence of pneumococcus serotype 3 was able to induce a higher activity of the NADPH oxidase at 10 μg/ml and 50 μg/ml, whereas at 100 μg/ml CRP induced a reduction in the function of this enzyme (Figure 3.21).

Some reports have claimed that isolation of CRP may contaminate the sample with either IgG or other proteins that are able to alter the outcome of the response obtained with CRP (Hundt *et al.*, 2001). However, pCRP when analysed in SDS-PAGE (see section 2.1.4) showed a single band (Figure 3.22), suggesting that only this protein was present in our preparation. We ruled out this possible problem of contamination with our purified CRP (pCRP) by doing some experiments with recombinant CRP (rCRP), which, because it is produced in *E. coli* from a single human gene, should not have any contaminating human protein.

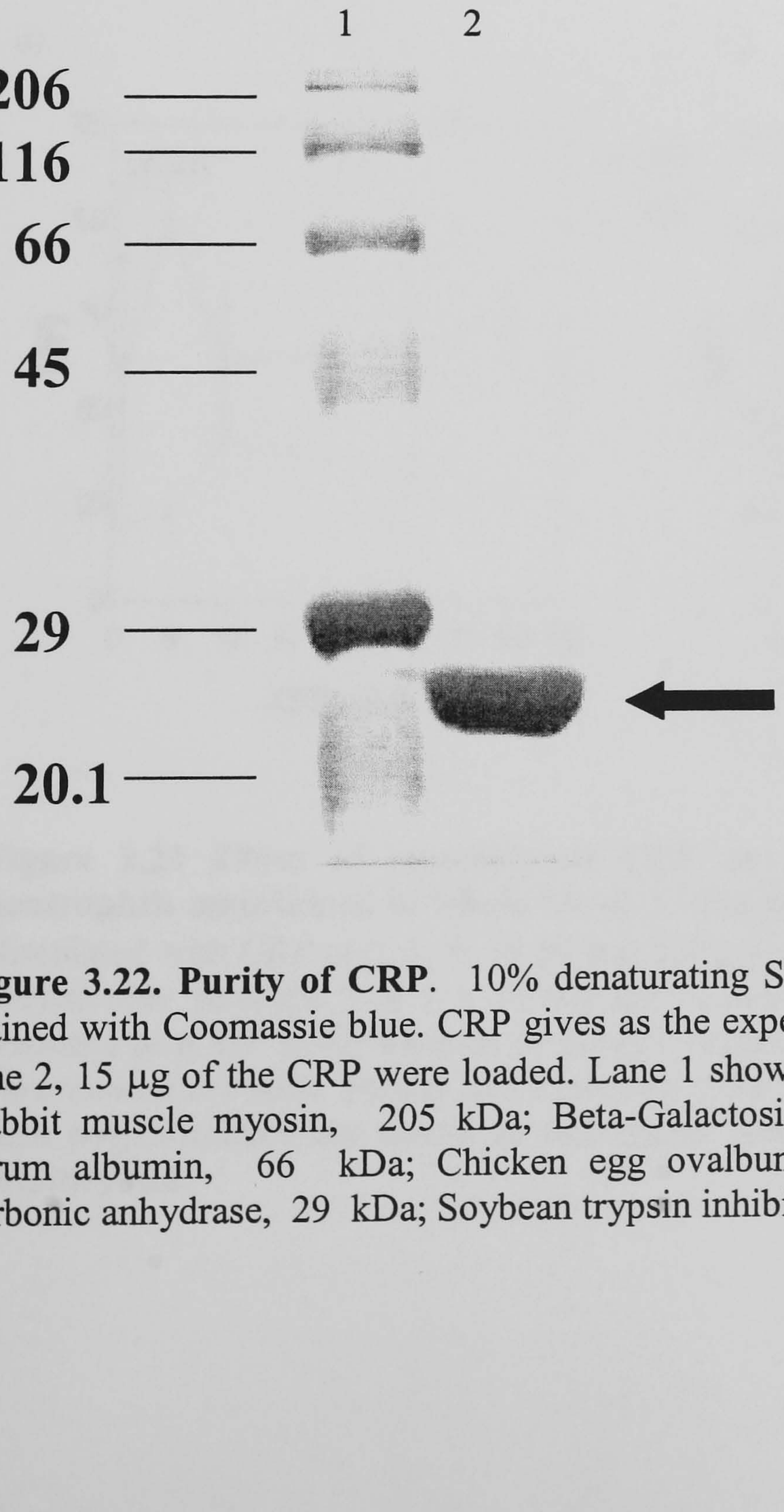


Figure 3.22. Purity of CRP. 10% denaturating SDS-PAGE of reduced CRP was stained with Coomassie blue. CRP gives as the expected band at 23 kDa (arrow). In lane 2, 15 μg of the CRP were loaded. Lane 1 shows the molecular weight markers. Rabbit muscle myosin, 205 kDa; Beta-Galactosidase, *E. coli* 116kDa; Bovine serum albumin, 66 kDa; Chicken egg ovalbumin, 45kDa; Bovine erythocyte carbonic anhydrase, 29 kDa; Soybean trypsin inhibitor, 20.1kDa.

We, therefore analysed NADPH oxidase activity in neutrophils from both HH and RR donors in the presence of various concentrations of rCRP and in the absence or presence of the type 3 pneumococcus. In panel b) of Figure 3.23 it can be observed that rCRP was able to induce effects similar to those obtained with the purified form of CRP; rCRP at 10 μg/ml was able to induce more H_2O_2 than in the absence or presence of rCRP at high concentrations (50 or 100 μg/ml). Panel a (without bacteria) only showed a significant increase at 5 μg/ml CRP. This result argues against a contaminating protein contained in the pCRP and also against a strong role of FcγRIIA in the function of CRP on neutrophils, since there was no evidence of CRP acting on RR rather than HH. Since these donors were normal volunteers it is expected that the blood contained 1 – 2 μg/ml of CRP.

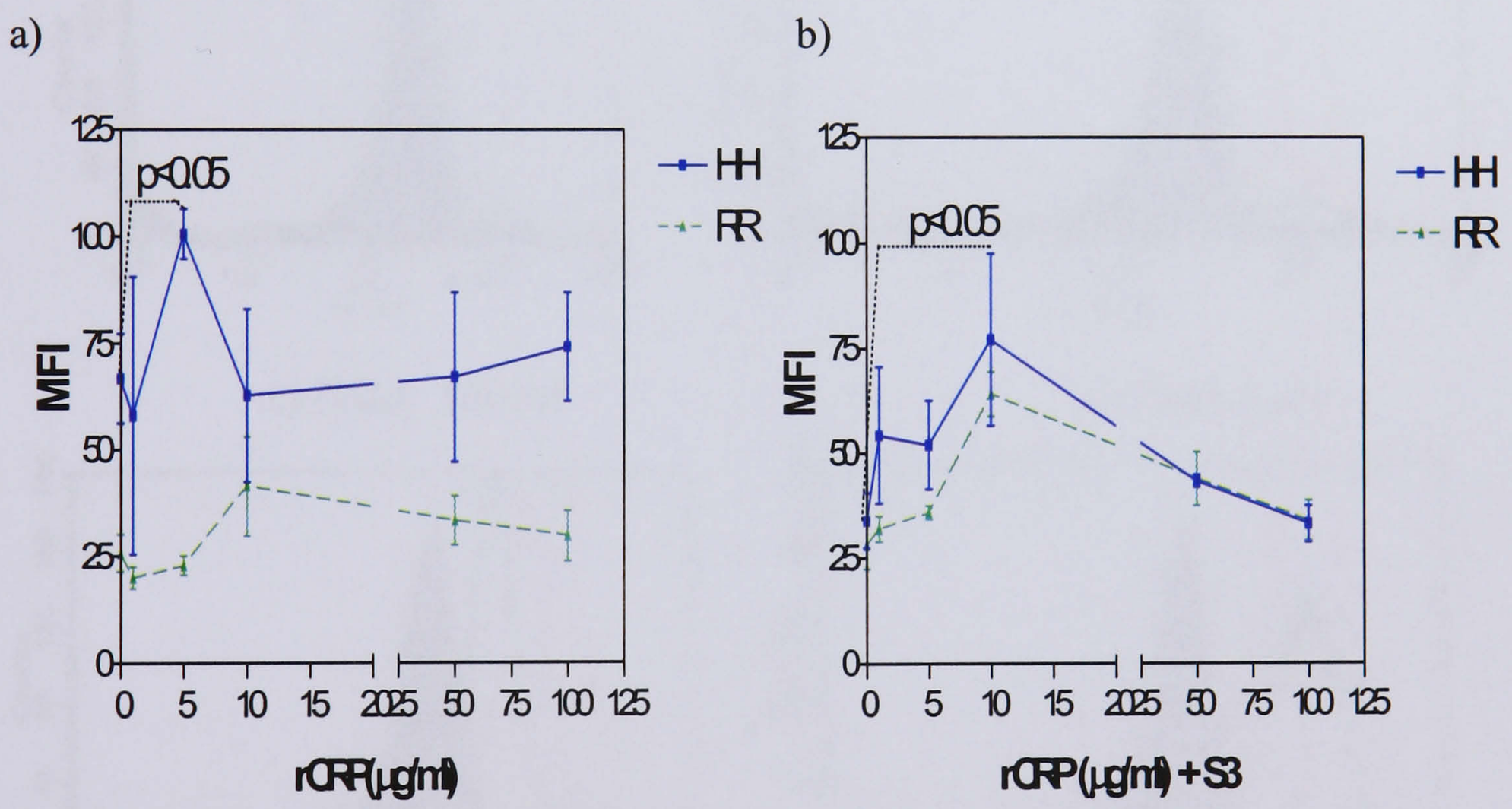


Figure 3.23 Effect of recombinant CRP on the oxidation of DHR 123 by neutrophils maintained in whole blood. Neutrophils from HH and RR donors were stimulated with CRP at 0, 1, 5, 10 50 and 100 μg/ml in the presence or absence of *S. pneumoniae* serotype 3 at 1 x 10^7/ml for 30 minutes at 37°C. Panel a shows the response to rCRP alone whereas in panel b responses to rCRP in the presence of *S. pneumoniae* are seen. Means and s.e.m. for neutrophils from 4 different donors for each polymorphism are shown in each panel. Data were analysed using the Mann Whitney test.

3.5.6 Increasing concentrations of rCRP alter NADPH oxidase activity according to the stimuli used.

Because CRP has been shown to modulate neutrophil NADPH oxidase activity induced by *S. pneumoniae*, we wished to establish whether CRP at increasing concentrations was also able to alter NADPH oxidase activity induced by other stimulants such as LPS, PMA and fMLP.

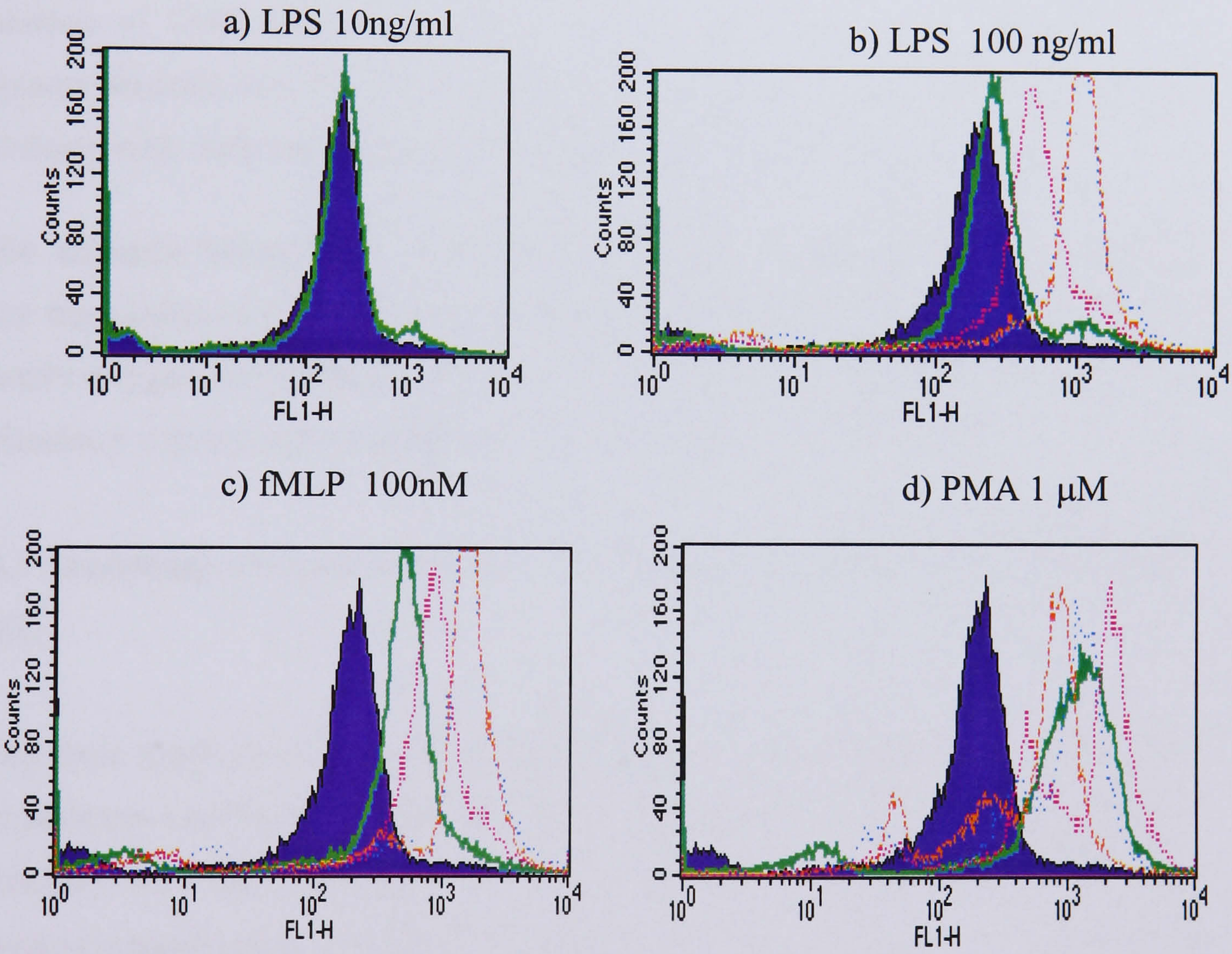


Figure 3.24. rCRP at increasing concentrations alters the NADPH oxidase activity on neutrophils according to the stimulant present. Neutrophils at 2 x 10^5 per condition were incubated with increasing concentrations of rCRP and different stimulants for 20 minutes at 37ºC and then DHR were added for a further 10 minutes. The reaction was stopped by adding 0.02% (w/v)EDTA, cells were fixed and acquired by FACS. In all cases cells alone with DHR are represented in the dark histogram. ——— agent alone, ····· CRP 1 μg/ml, ····· CRP10μg/ml and – – CRP 100 μg/ml. Two different experiments showed similar results.

Since rCRP contains about 10 ng/ml of contaminating LPS we first examined whether LPS at this concentration could alter neutrophil NADPH oxidase activation and affect DHR oxidation. However, at this concentration LPS was not able to induce any effect on DHR as shown in panel a of Figure 3.24, higher concentrations of LPS such as 100 ng/ml were able to induce weak DHR oxidation as shown in panel b of Figure 3.24.

Interestingly, rCRP at increasing concentrations was able to down-regulate the oxidation of DHR induced by PMA (panel d Figure 3.24) but upregulated the response obtained with fMLP and LPS (panels b and c of Figure 3.24), which may be correlated with different pathways activated by these different neutrophil stimulants.

PMA activates through PKC and leads to calcium and IP_3 signaling. In addition, since the calcium ionophore ionomycin can activate the intracellular production of reactive oxygen, we tested the ability of CRP to inhibit this response. However, the preliminary experiments suggest that CRP did not reduce the reponse.

3.5.7 Increasing concentrations of CRP augment the amount of extracellular H_2O_2

Since there might be differences in the production of H_2O_2 between the intracellular compartment and the extracellular medium, we compared the synthesis of hydrogen peroxide by the detection of resorufin derived from 10-acetyl-3,7-dihydroxyphenoxazine. Purified CRP was used at 1, 5, 10, 50 and 100 μg/ml in isolated neutrophils to analyse the effect of this protein in the presence of the pneumococcus serotype 3. Clearly at increasing concentrations of pCRP more H_2O_2 was produced (Figure 3.25).

This result differs from those obtained measuring intracellular H_2O_2 and suggests that different mechanisms control the production of radicals of oxygen inside or outside the phagocytic cell.

3.5.8 Superoxide production induced by recombinant CRP in isolated neutrophils from both HH and RR donors.

To check whether recombinant CRP was also able to induce similar changes in the production of superoxide by neutrophils, neutrophils from both HH and RR donors were cultured in the presence of rCRP at increasing concentrations. Determination of reduction of membrane non-permeable cytochrome c was performed at different time points over approximately 2 hours as shown in Figure 3.26 with superoxide production expressed as nmol / 10^5 cells as in previous reports.

PMA was used as a positive control and gave the maximum value; rCRP at increasing concentrations induced higher production of superoxide. This was similar to the pattern of synthesis of extracellular H_2O_2 described in the previous section. Both HH and RR donors gave a similar result. Phagocytosis and reactive oxygen responses and others are reported to be induced through FcγRIIA. Thus if as reported (Stein et al., 2000b) CRP binds to R allele but not H allele these results argue against a major role of FcγRIIA in the response of neutrophils to CRP. An alternative explanation may be that CRP binding to R and H alleles shows less difference in avidity than previously reported particularly if acting in a complex with other receptors. Since this latest experiment was performed using rCRP it was unlikely that other contaminating proteins altered the responses.

The presence of *Streptococcus pneumoniae*, which in preliminary results gave us low amounts of superoxide, was avoided in this assay since its presence can interfere with the assay by the accumulation of H_2O_2 which can reoxidise cytochrome C and therefore confound the result obtained (Dahlgren and Karlsson, 1999).

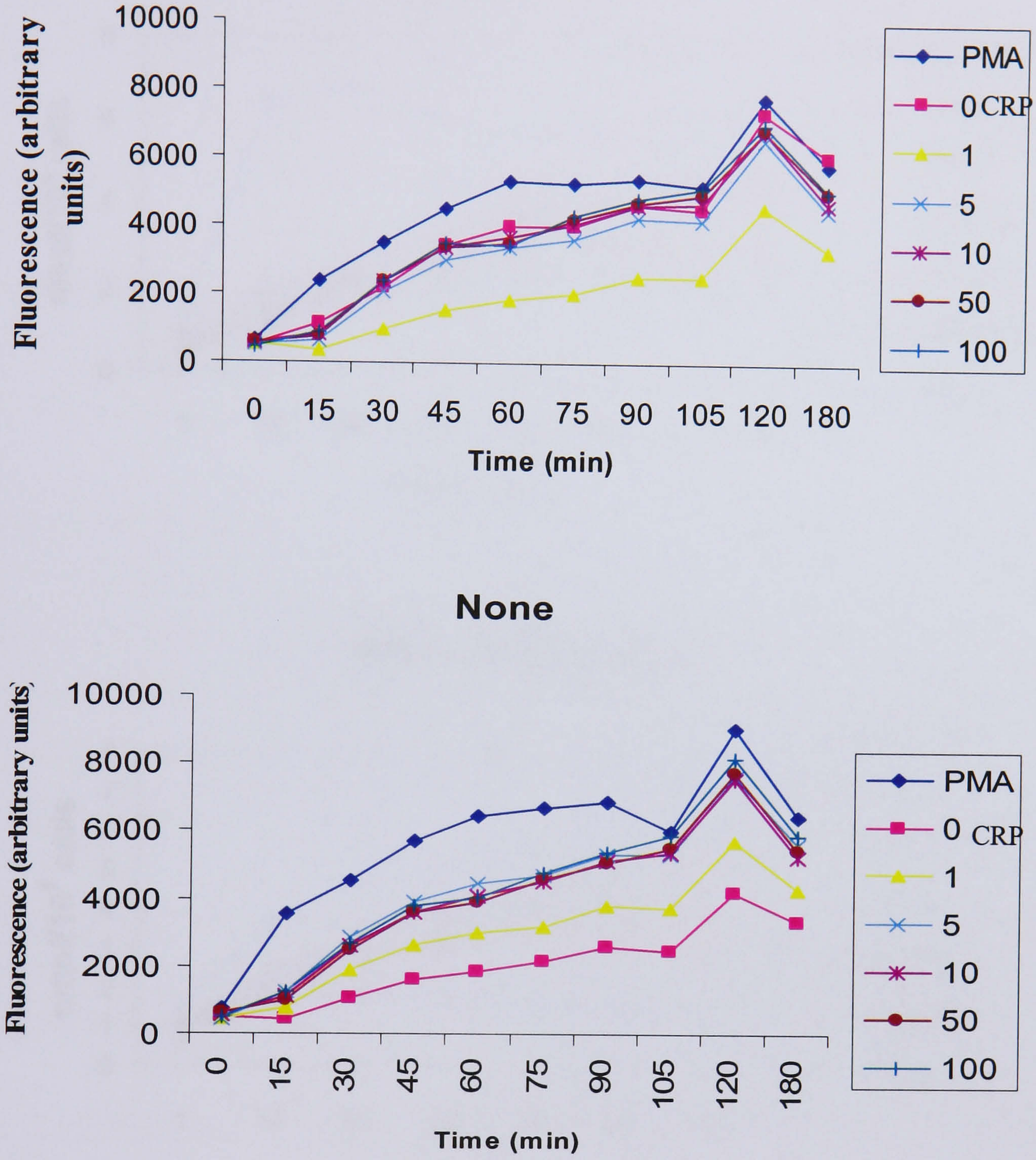


Figure 3.25. CRP increases the production of extracellular H_2O_2 by neutrophils. 1.5×10^4 isolated neutrophils were incubated for 3 hours with or without *Streptococcus pneumoniae* at a ratio of 30:1, in the presence of purified CRP at 0, 1, 5, 10, 50 and 100 μg/ml. Each 15 minutes fluorescence of amplex red reagent (methods section 2.7.3.3) was measured using excitation at 560nm and emission at 690 in a spectrofluorometer reader. One experiment was performed to check for the extracellular production of H_2O_2.

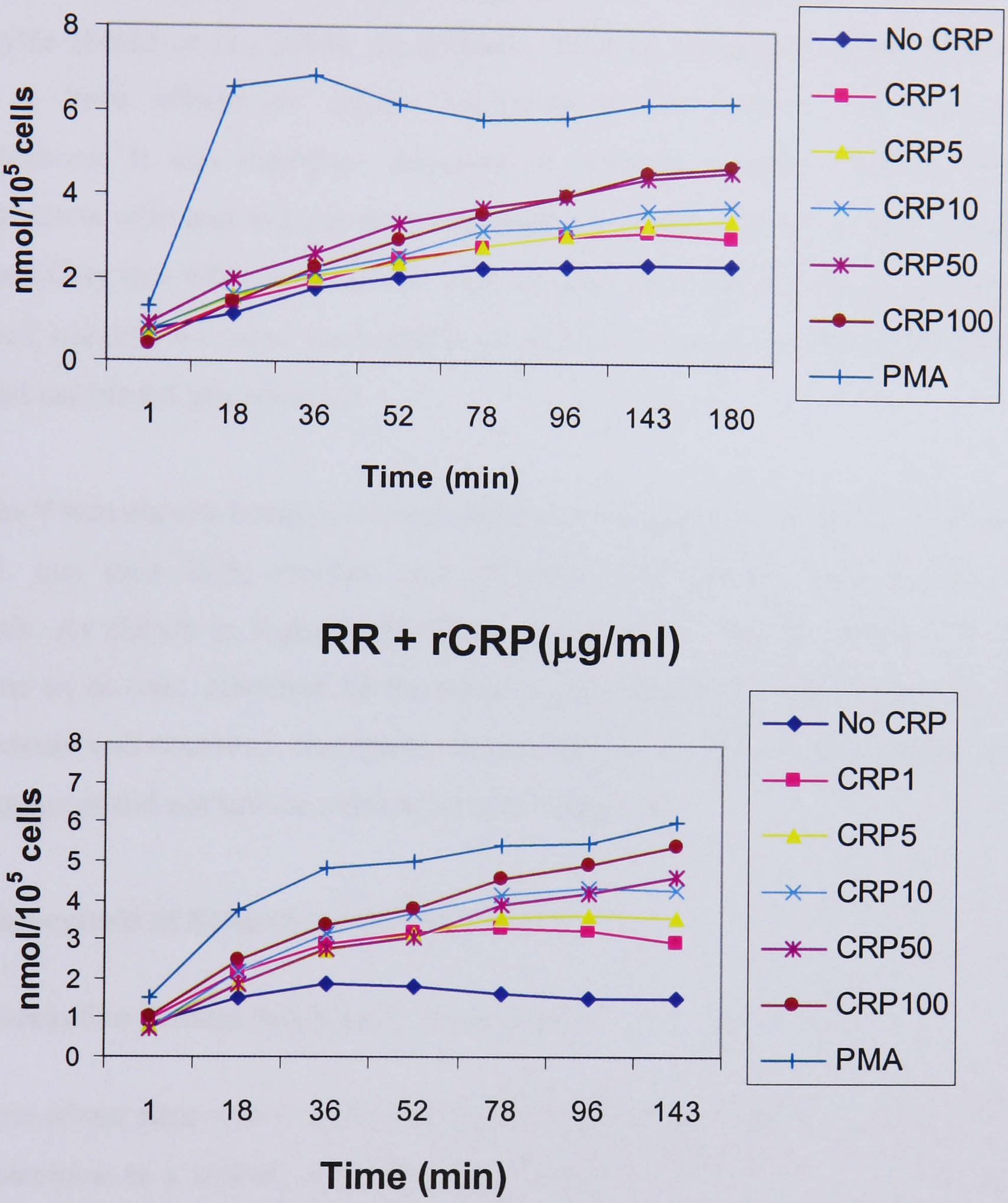


Figure 3.26. Production of superoxide anion by reduction of cytochrome c by neutrophils. 10^5 neutrophils from both HH and RR individuals were incubated for approximately two hours with recombinant CRP at 0, 1, 5, 10, 50 and 100 μg/ml at 37ºC. Cytochrome c reduction was followed by absorbances at 550 nm in a microplate spectrofluorimeter. The amount of superoxide produced is expressed as nmol/10^5 cells, calculated according to the formula described in section 2.7.3.4. Two independent experiments from two different donors gave similar results.

3.5.9 Effect of CRP and *S. pneumoniae* on the neutrophil apoptosis

CRP has been reported to promote opsonisation of apoptotic neutrophils by phagocytes (Mold *et al.*, 2002). In addition, CRP in subsequent experiments was shown to have effects on certain responses at intermediate and acute-phase concentrations. It was important therefore to examine whether CRP at different concentrations with and without the pneumococcus could alter neutrophil apoptosis. The possibility that MBL was able to alter the rate of neutrophil apoptosis was not examined because we were particularly interested in opsonin coated particles and MBL did not bind *S. pneumoniae*.

Annexin V was chosen because it measures the early stages of apoptosis and because TUNEL can stain both necrotic and apoptotic cells giving false positives for apoptosis. As shown in Figure 3.27 CRP appears not to induce further neutrophil apoptosis on its own. However, in the presence of pneumococci increasing apoptosis and necrosis was observed. Increasing concentrations of CRP in the presence of the pneumococcus did not induce more neutrophil apoptosis.

3.6 Phagocytosis of *Streptococcus pneumoniae*

3.6.1 C-reactive protein binds to *S. pneumoniae*

Since one of our aims was to study the interaction between neutrophils and CRP with *S. pneumoniae* as a model, we investigated whether CRP binds to pneumococcus serotype 3 which by having a capsule might not be expected to be a good binder. In figure 3.28 panel a, antibody to CRP binds better than the isotype control at 1/50 and 1/100. Since 1/100 dilution showed higher binding this dilution was chosen for the experiment illustrated in panel b, in which CRP bound better at 50 and 100 μg/ml although more diffuse binding was seen.

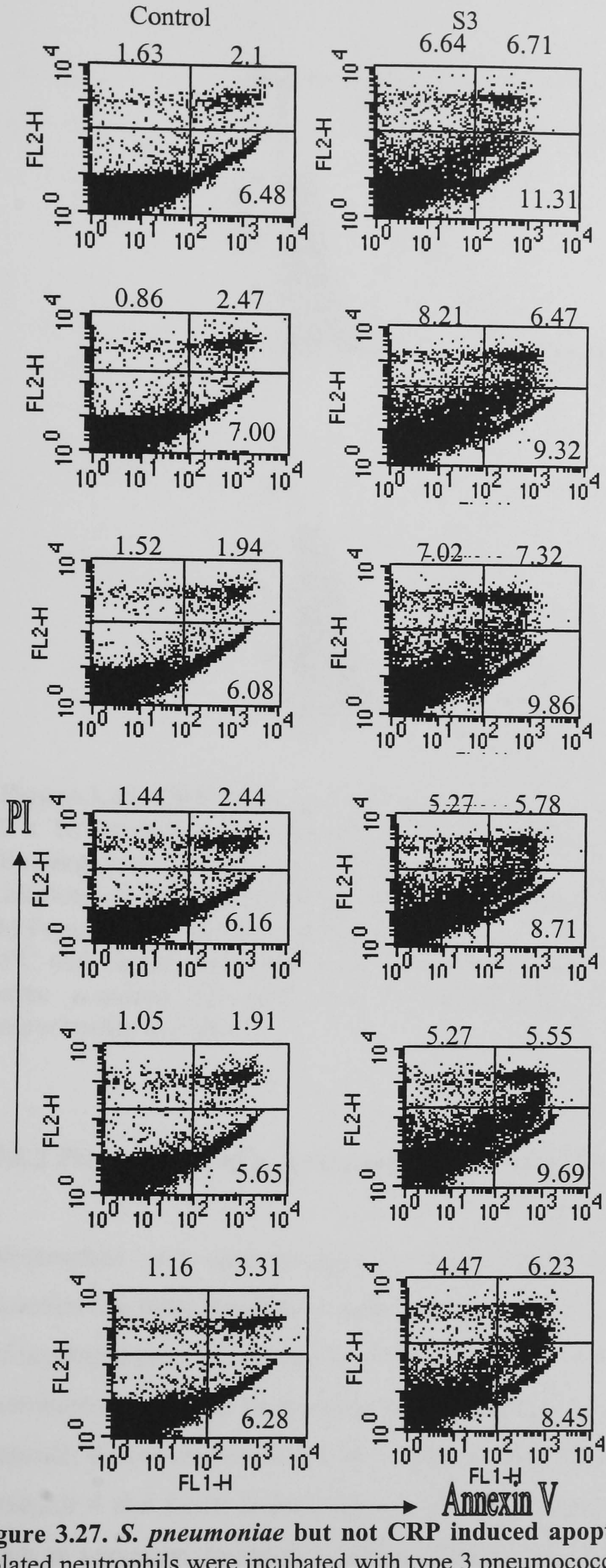
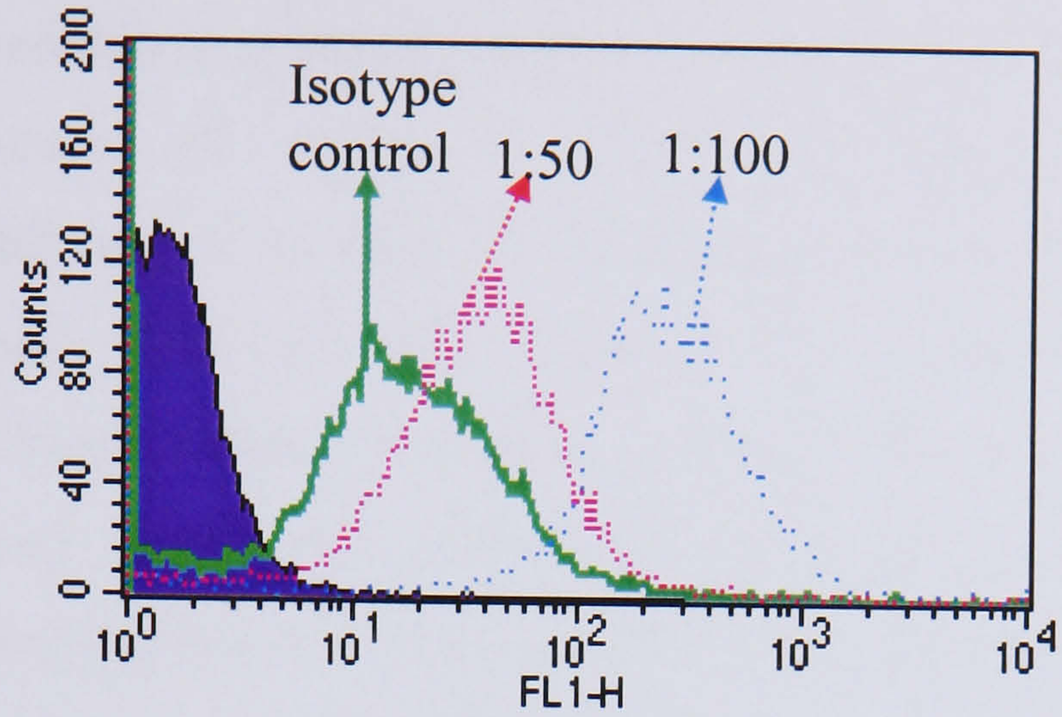


Figure 3.27. *S. pneumoniae* but not CRP induced apoptosis of neutrophils. 1 x 10^6/ml isolated neutrophils were incubated with type 3 pneumococcus for 8 hours in the presence of different concentrations of pCRP. Cells were stained with annexin V (FL1) and propidium iodide (FL2) as described in section 2.3.1. The percentages of cells stained with one or both reagents are shown. Neutrophils from two donors gave similar results.

a)



b)

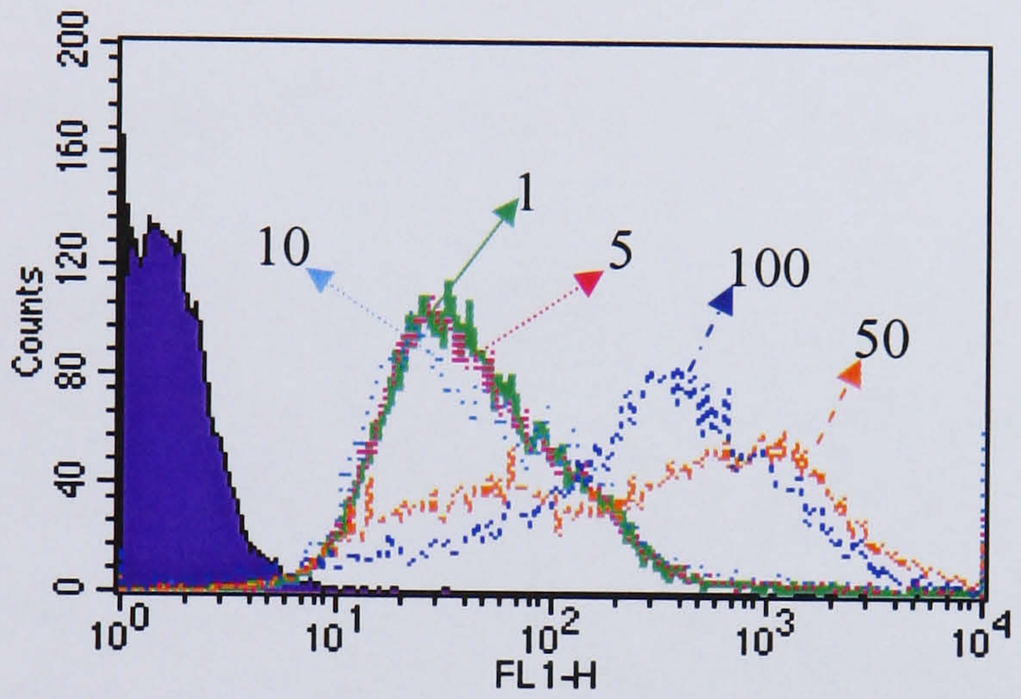


Figure 3.28. CRP binds to *S. pneumoniae* type 3. a) CRP at 25μg/ml was added to 2 x 10^6 pneumococcus for 1 hour at 4ºC. Filled histograms show bacteria alone. Binding was detected by a mouse anti-human CRP antibody at 1/50 and 1/100 dilutions as described in section 2.5.4 and it was compared with the isotype control. b) Pneumococci were incubated with CRP at 1, 5, 10, 50 and 100 μg/ml for 1 hour at 4ºC and binding detected using 1/100 dilution of antibody. At least 30000 events were acquired for each test. A representative histogram from two different experiments is shown.

3.6.2 Phagocytosis of *S. pneumoniae* by macrophages

Neutrophils and macrophages are professional phagocytes and this particular function has been analysed in different ways. We have chosen to examine the ability of macrophages to phagocytose FITC- labelled *S. pneumoniae*. Although usually the pneumococcus has to be opsonised to be phagocytosed because of the presence of the capsule, this bacterium might be phagocytosed directly by contact with the toll like receptor 4 that binds to proteoglycan or lipoteichoic acid or by scavenger receptors such as scavenger receptor A, which is expressed on macrophages and allows them to phagocytose *S. pneumoniae* without opsonisation.

CRP which took its name from the ability to bind to the C-polysaccharide of *Streptococcus pneumoniae* is expected to opsonise pneumococcus and to increase its phagocytosis. However, the effects of different concentrations of CRP and comparisons of pCRP and rCRP have not been previously analysed. To show the ability to phagocytose CRP-opsonised pneumococcus mononuclear cell derived macrophages were chosen because of their size. Opsonisation of *S. pneumoniae* type 3 with CRP induced more phagocytosis by mononuclear cell derived human macrophages when compared to non-opsonised bacteria (Figure 3.29).

3.6.3 Phagocytosis of *S.pneumoniae* by human neutrophils

Phagocytosis of *S. pneumoniae* by neutrophils was first demonstrated by confocal microscopy in collaboration with Rachel Gregory. Neutrophils were able to phagocytose low numbers of pneumococci without any opsonisation but many more when pneumococci were opsonised with normal human serum (Figure 3.30). In the same way CRP-opsonised pneumococci serotype 3 were also phagocytosed by neutrophils. Figure 3.31 clearly shows that the microorganisms are inside the cell in the scan shown at the bottom.

Figure 3.32 (panel a) shows that purified CRP at 30μg/ml was able to induce phagocytosis of pneumococcus type 3 at different neutrophil: pneumococcus ratios. Phagocytosis could be inhibited at 4°C when compared to 37°C (panel b) and also in the presence of the F-actin disrupting drug cytochalasin D (Figure 3.33). A decrease in the phagocytosis of pneumococci was obtained with high concentrations of CRP (50 or 100 μg/ml) compared with 10 μg/ml as can be seen in Figure 3.34, regardless of the type of polymorphism of the FcγRIIA.

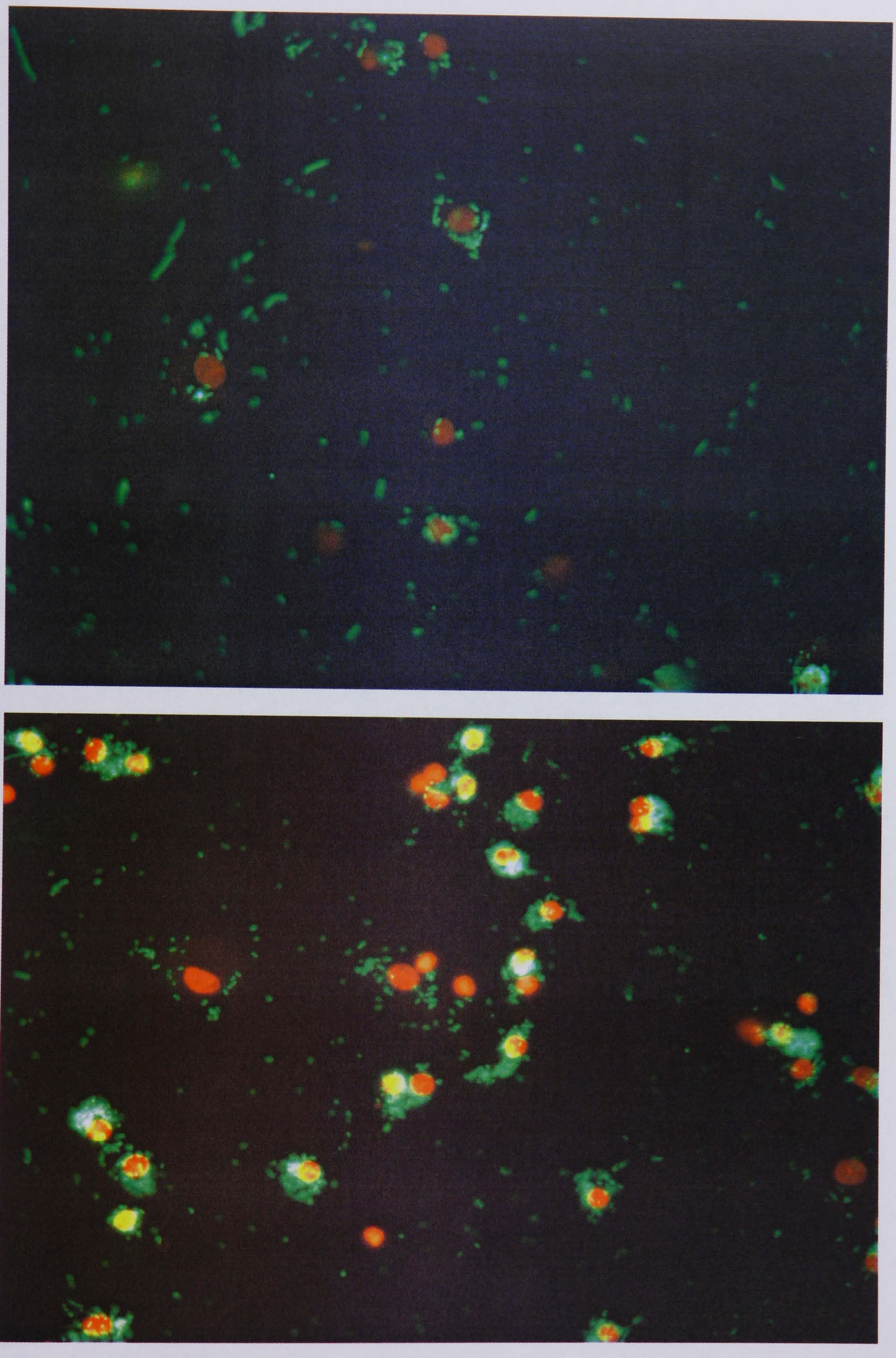


Figure 3.29. Phagocytosis of rCRP opsonised–*S. pneumoniae* type 3 by macrophages. 4 x 10^5 adherent PBMCs were cultured for 5 days in the presence of RPMI and 10% FCS at 37°C. At day 6, FITC labelled S*treptococcus pneumoniae* in the absence (upper picture) or presence of 30 μg/ml rCRP (lower picture) at a ratio 30:1 were added and incubated for 1 hour at 37°C. Cells were counter-stained with ethidium bromide and visualised by fluorescent microscopy.

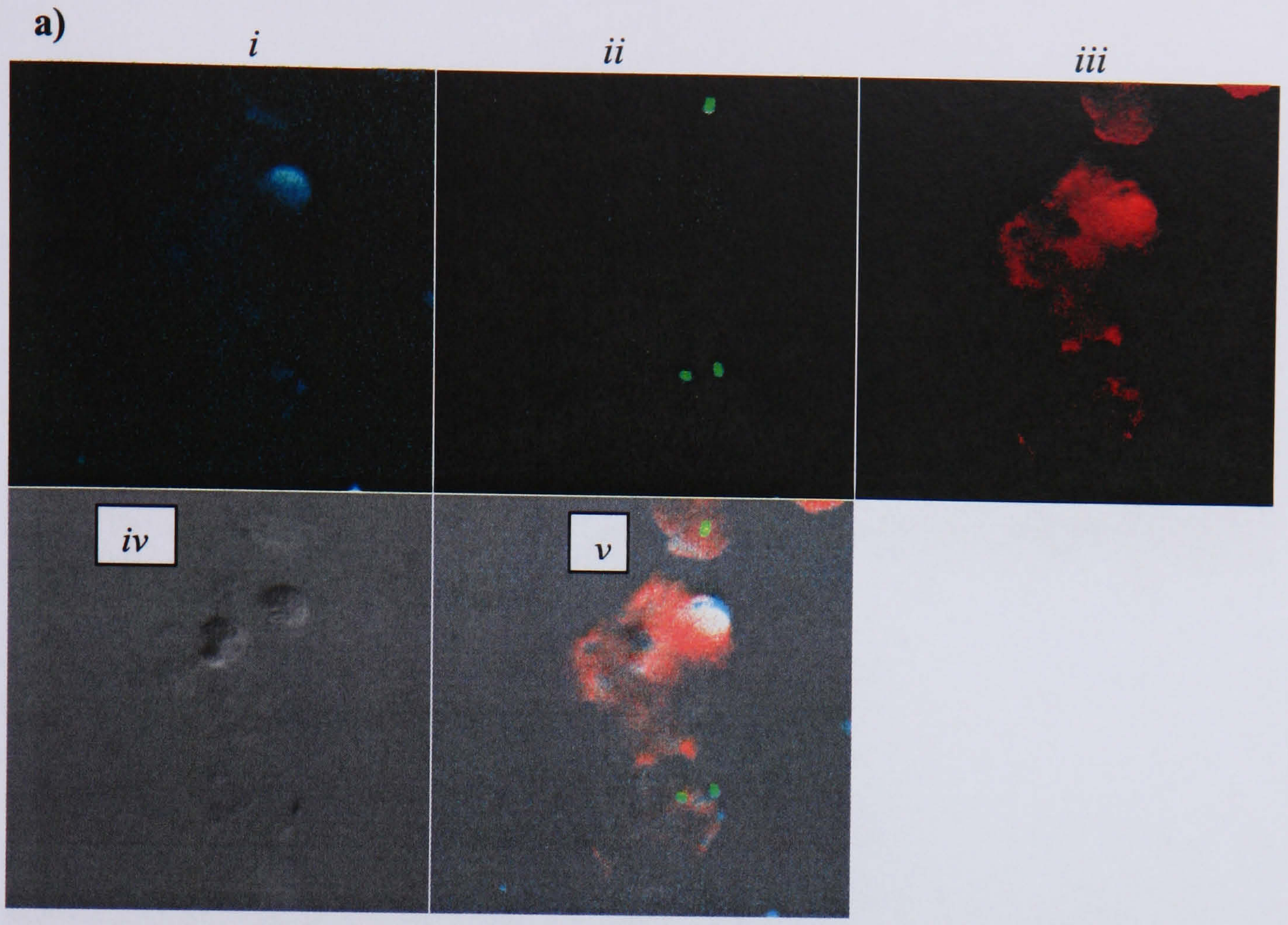


Figure 3.30. Phagocytosis of *S. pneumoniae* by neutrophils. 2 x 10^5 neutrophils were incubated with FITC-labeled *S. pneumoniae* serotype 3 in a ratio 1:30 (neutrophil: pneumococci) for 30 minutes at 37°C. After washing, methanol fixation and staining with phalloidin (blue staining) and ethidium bromide stained cells were visualised by confocal microscopy. Figure group a) shows control pneumococci and figure group b) shows pneumococci pre-opsonised with 10% normal human serum. Panel (*i*) shows phalloidin staining, (*ii*) FITC-labelled pneumococci, (*iii*) ethidium bromide staining, (*iv*) Differential interference contrast (DIC) image and (*v*) a merged image of the previous panels. This experiment was performed in collaboration with Rachel Gregory.

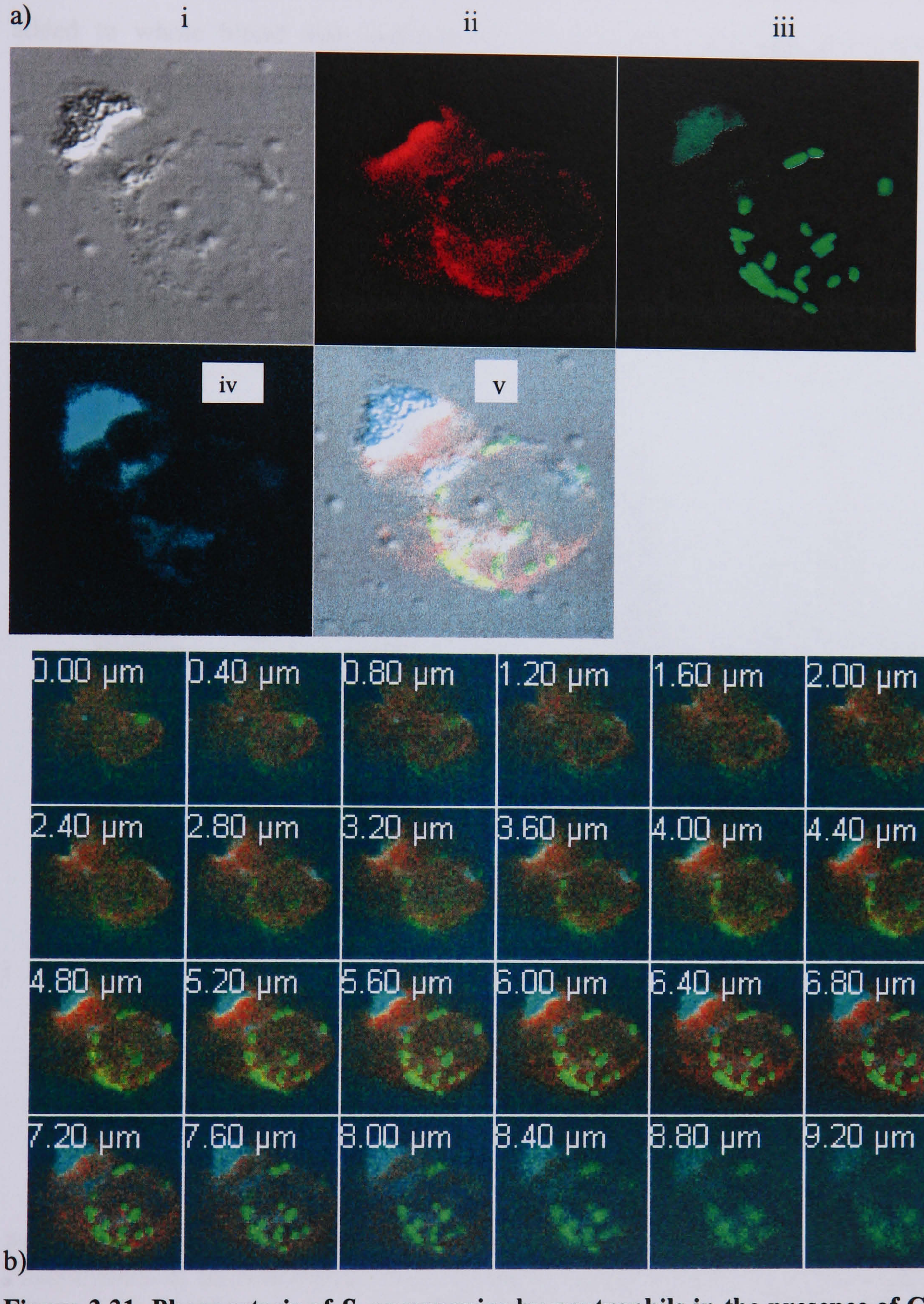


Figure 3.31. Phagocytosis of *S. pneumoniae* by neutrophils in the presence of CRP. 2×10^5 neutrophils were incubated with FITC-labeled *S. pneumoniae* serotype 3 preopsonised with 30 µg/ml rCRP in a ratio 1:30 (neutrophil: pneumococci) for 30 minutes at 37°C on glass slides. After methanol fixation and staining with phalloidin (blue staining) and EB-stained cells were visualised by confocal microscopy. Figure group a) shows control pneumococci in which (*i*) is the DIC image, (*ii*) ethidium bromide staining, (*iii*) FITC-labelled pneumococci, (*iv*) phalloidin staining and (*v*) is the merge image. Figure group b); z-stack of the phagocytosing cells is shown from the top (0.00μm) to the bottom of the cell (9.40 μm). This experiment was performed in collaboration with Rachel Gregory.

Phagocytosis of *Streptococcus pneumoniae* by neutrophils in the presence of rCRP added to whole blood was maximal at 10 ug/ml and increased concentrations appeared toresult in slightly reduced uptake (Figure 3.35). In these experiments rCRP was employed since possible contaminants of pCRP may inhibit phagocytosis of pneumococci by blocking particular receptors.

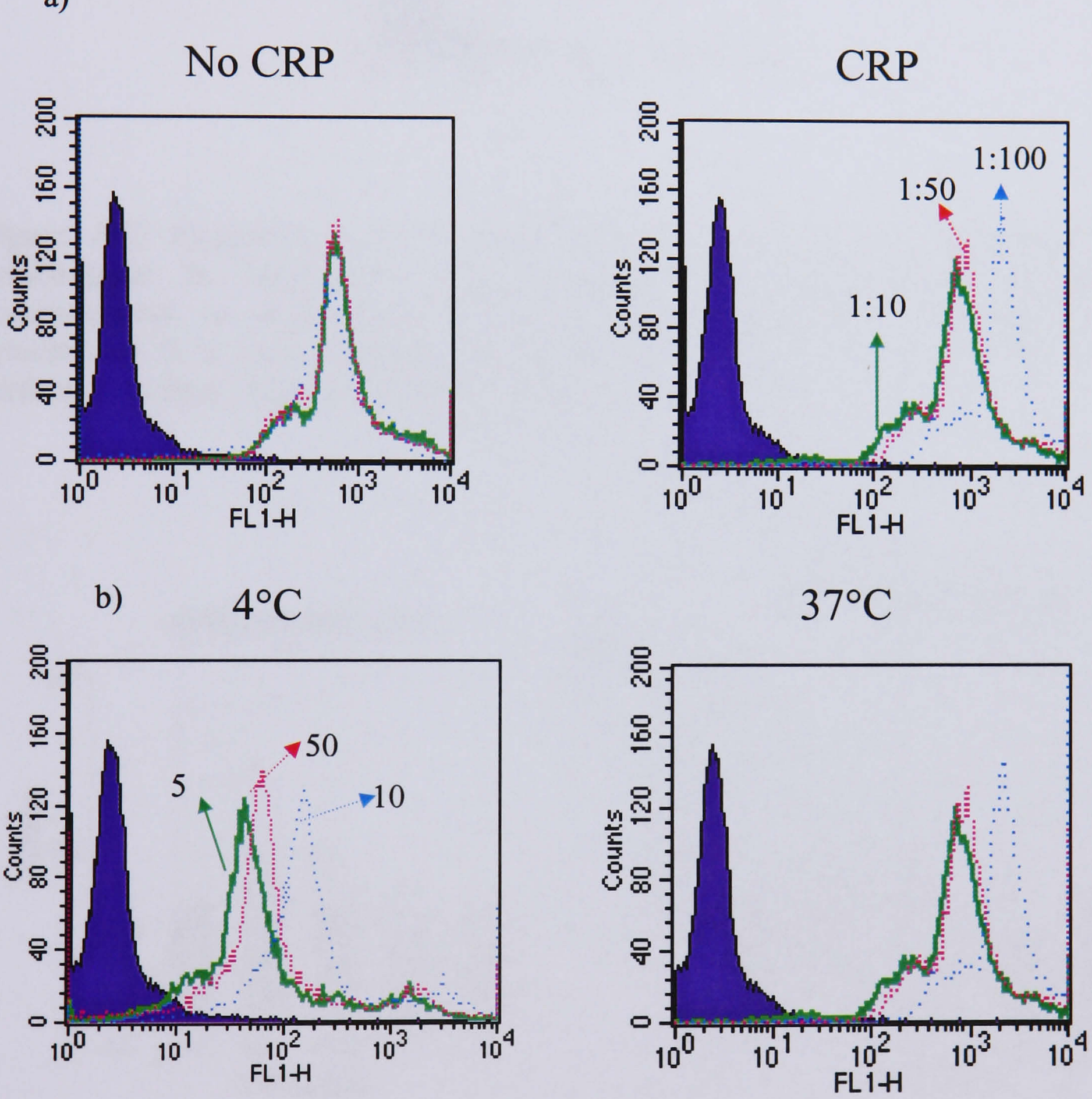


Figure 3.32. Phagocytosis of *S. pneumoniae* by isolated neutrophils. a) Phagocytosis of pneumococcus at 37ºC for 1 hour were performed using different ratios of neutrophils: bacteria: 1:10, 1:50, 1:100 in the presence of 30μg/ml CRP b) two groups of 2 x 10^6/ml neutrophils were incubated with *S. pneumoniae* at 4ºC for 30 minutes and then one of them was transferred to a 37ºC for a further period of 30 minutes. Both groups were incubated in the presence of CRP at 5, 10 and 50 μg/ml.

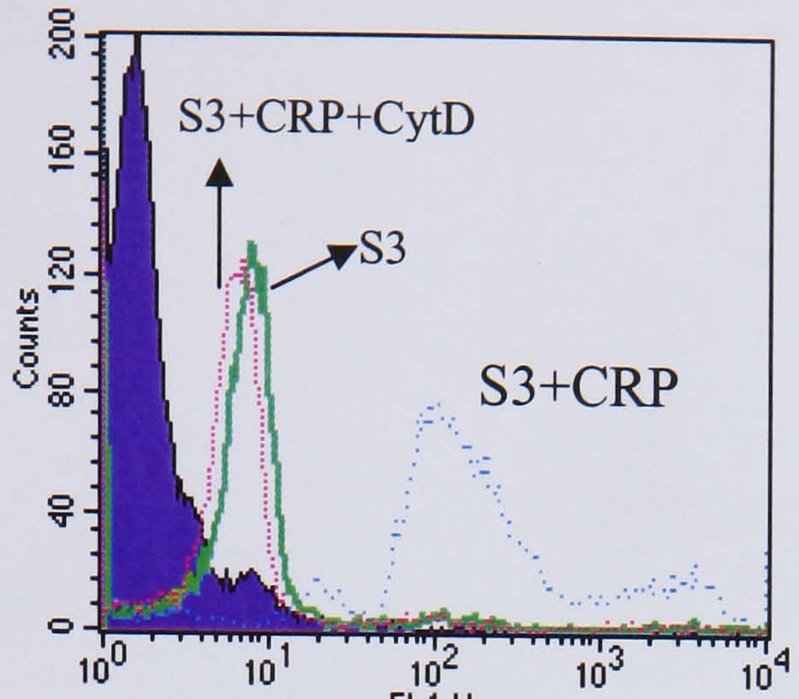


Figure 3.33 Phagocytosis of *S. pneumoniae* by neutrophils is inhibited by cytochalasin D. Neutrophils were incubated with 30μg/ml CRP-opsonised pneumococcus, as described in section 2.7.4.2 in the absence or presence of cytochalasin D or with non-opsonised pneumococcus type 3. One experiment was performed to show inhibition by cytochalasin D.

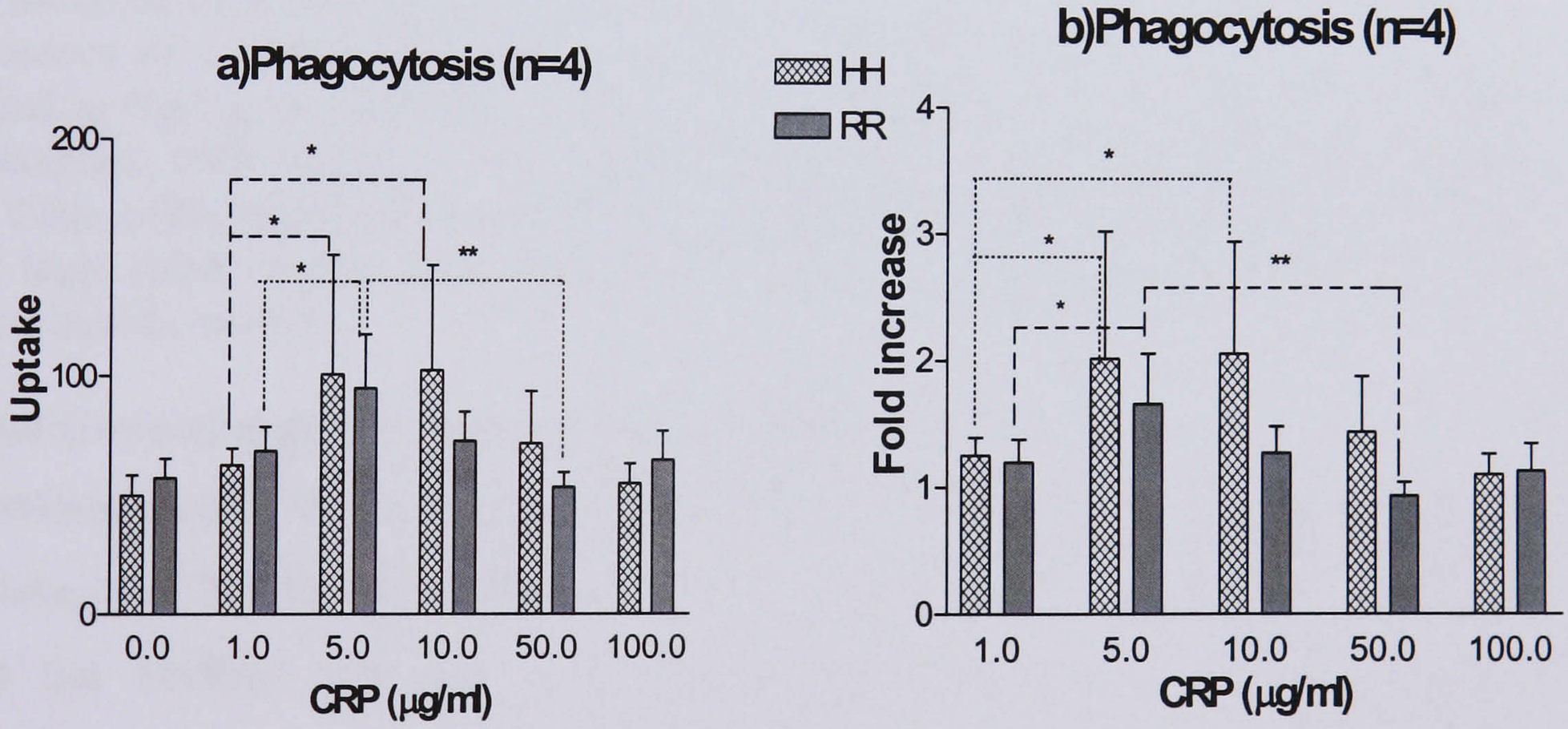


Figure 3.34. Phagocytosis of *S. pneumoniae* is decreased at high concentrations of CRP in both HH and RR individuals. a) Phagocytosis of pneumococci at a ratio 1:30 (cells: pneumococci) was performed by 2 x 10^6 neutrophils/ml during 30 minutes incubation at 37°C in the presence of different concentrations of purified CRP: 1, 5, 10, 50 and 100 μg/ml. To determine uptake the MFI difference between each CRP concentration and that obtained by adding cytochalasin D was obtained. b) Fold increase over the baseline for each individual is shown for both HH and RR donors. Data were analysed using Mann Whitney test. * $p<0.05$; ** $p<0.01$.

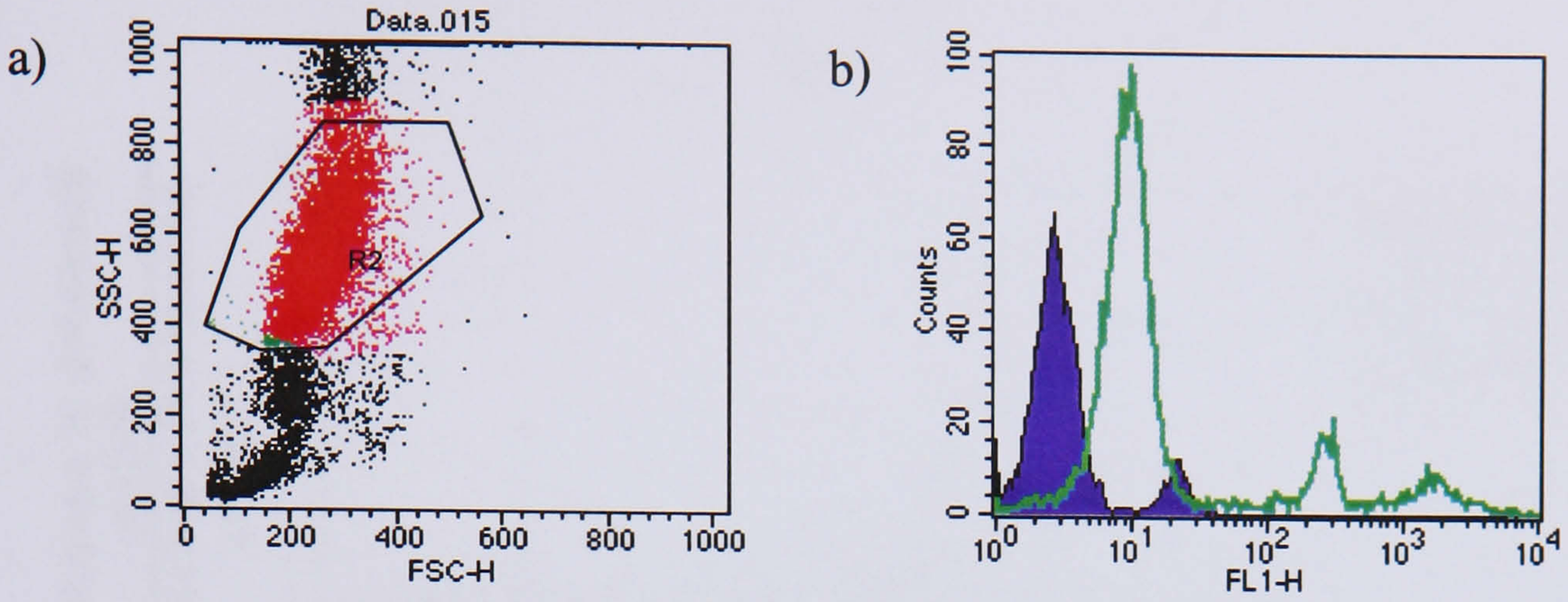


Figure 3.35. Phagocytosis of *S. pneumoniae* by neutrophils in whole blood. 100 µl of blood were incubated for 30 minutes at 37ºC with 2 x 10^6 *S. pneumoniae* in the presence of rCRP as described in the Materials and Methods section 2.7.4.3 . In panel a) Gating of neutrophils is shown. b) Cells alone are represented in the filled histogram, whereas the open histogram shows cells with pneumococci alone and in c) Cells with pneumococci and rCRP at 0 (filled histogram), 10, 50 and 100 µg/ml. At least 10000 events were acquired by FACS in three different experiments that gave similar results.

3.6.4 C-reactive protein and transfected Cos cells

Previous studies shown in section 1.3.2.2 demonstrate that CRP could increase the uptake of PCh labelled SRBC into FcγRIIA RR and FcγRI co-transfected Cos cells but not FcγRIIA HH and FcγRI co-transfected Cos cells. We attempted to demonstrate that the same could be observed for *S. pneumoniae* rather than SRBC, because bacteria are a smaller particle and might be able to alter neutrophils by different mechanisms. Due to high non-specific binding of pneumococci to Cos cells (30% of vector-only transfected cells showed binding) we have been unable to confirm any role for this receptor in the phagocytosis of CRP-opsonised pneumococci (Figure 3.36). Several washing steps were introduced in an attempt to reduce background but none were successful.

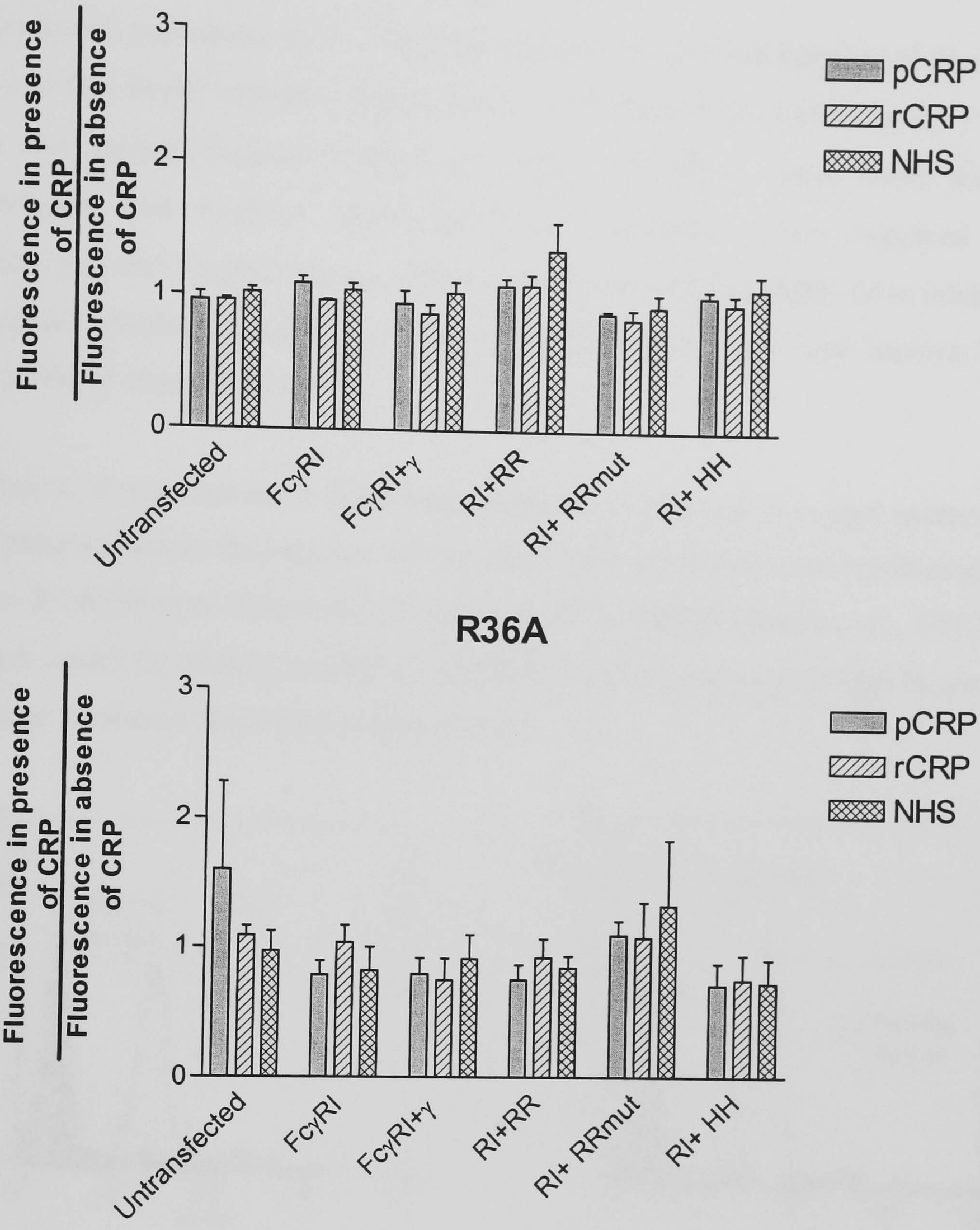


Figure 3.36. *S. pneumoniae* association to transfected Cos cells. Cos cells were transfected with Syk and with FcγRI alone; FcγRI + γ chain; FcγRI + FcγRIIA RR; FcγRI + FcγRIIA RR mutant and FcγRI + FcγRIIA HH. *S. pneumoniae* was preincubated with 30 μg/ml of either rCRP or pCRP and after washing, phagocytosis was allowed to proceed for 2 hours at 37°C. FACS analysis was performed to determine fluorescence of Cos cells in the presence and absence of CRP. Bars show means and s.e.m. of five different experiments. No significant difference was observed between the different conditions when data were analysed by Mann Whitney test.

3.7 Expression of FcγRIIB by neutrophils

Experiments performed in Dr J Raynes's laboratory on FcγR-transfected Cos cells showed that FcγRI increased phagocytosis of CRP-opsonised SRBC, when γ chain was also present (Bodman-Smith *et al.*, 2002). Interestingly when FcγRI was co-transfected with FcγRIIA, phagocytosis was stimulated when compared with transfection with FcγRIIA alone, while co-transfection with FcγRIIB led to inhibition of uptake (Bodman- Smith K., Gregory R., Rodriguez J. A. and Raynes J. G. Unpublished observation).

Therefore, it was relevant to determine whether reduced reactive oxygen species and low phagocytosis in the presence of high CRP concentration by neutrophils might be due to FcγRIIB since these cells do express FcγRIIB mRNA (Cassel *et al.*, 1993). To further search for binding of CRP to FcγRIIB, we performed experiments by surface plasmon resonance (described in section 2.8).

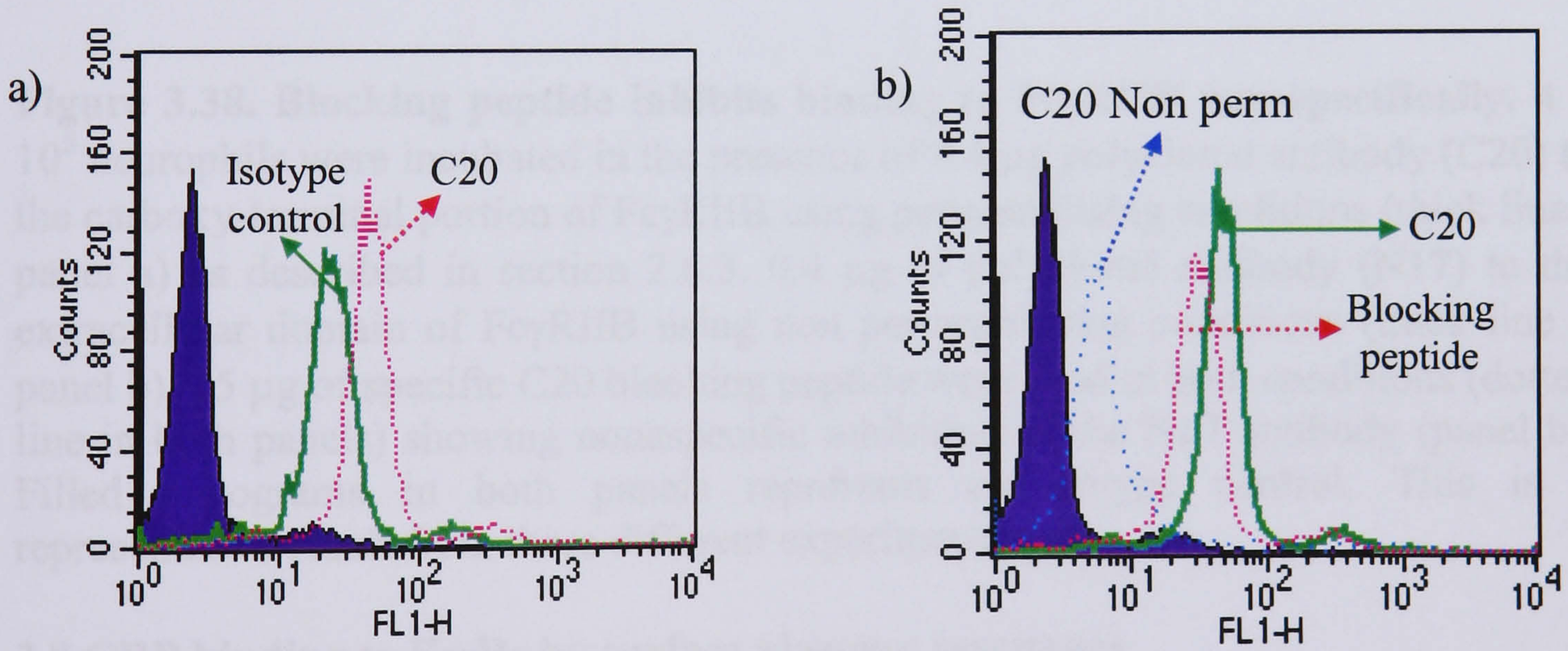


Figure 3.37. Expression of FcγRIIB on neutrophils. a) 4 x 10^5 neutrophils were incubated alone (filled histogram) or in the presence of 0.4 μg polyclonal antibody (C20) to the carboxy-terminal portion of FcγRIIB or with an isotype control, using permeabilising conditions for 1 hour at 4°C b) Neutrophils alone (filled histogram) or incubated with C20 using permeabilised or non-permeabilised conditions. 25 μg of specific C20 blocking peptide were also used as described in section 2.6.3. At least 10000 events were acquired by FACS. Two independent experiments gave similar results.

FcγRIIB appeared to be expressed on neutrophils using a polyclonal antibody to the carboxy-terminal portion of the FcγRIIB (Figure 3.37 panel a). This expression was inhibited by a specific blocking peptide (panel a). However, peptide could inhibit binding of antibody to a different epitope (Figure 3.38 panel b) when the binding site was not accessible. Therefore a different strategy or better antibody is needed to corroborate the expression of FcγRIIB on neutrophils.

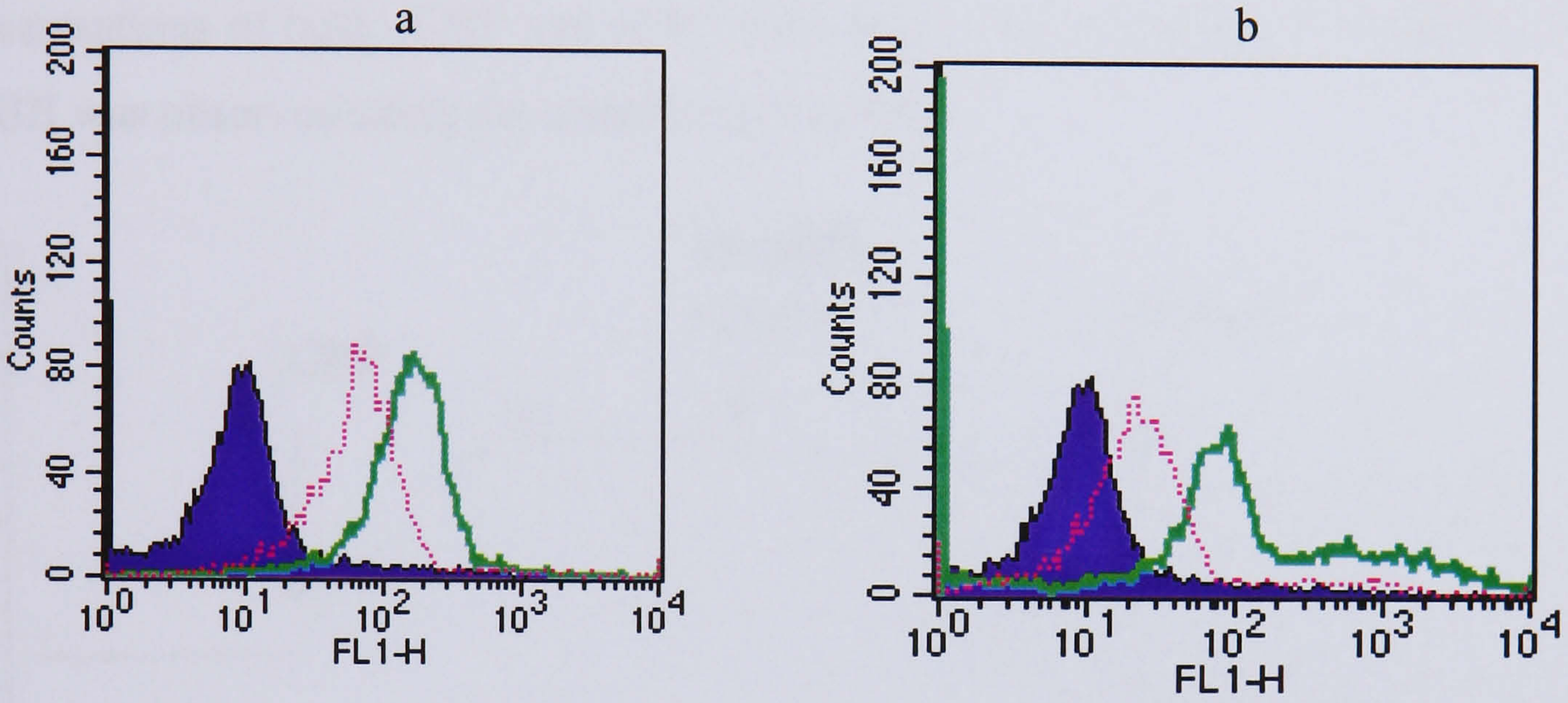


Figure 3.38. Blocking peptide inhibits binding to FcγRIIB non-specifically. 4 x 10^5 neurophils were incubated in the presence of 0.4 μg polyclonal antibody (C20) to the carboxy-terminal portion of FcγRIIB using permeabilising conditions (thick line - panel a) as described in section 2.6.3. 0.4 μg of polyclonal antibody (N17) to the extracellular domain of FcγRIIB using non permeabilising conditions (thick line – panel b). 25 μg of specific C20 blocking peptide were used in both conditions (dotted line in both panels) showing nonespecific inhibition of the N17 antibody (panel b). Filled histograms in both panels represents an isotype control. This is a representative result from three different experiments.

3.8 CRP binding to FcγRs by surface plasmon resonance

CRP has been recently shown to bind in a calcium dependent way to FcγRI using the surface plasmon resonance (BIACORE) method which allows the calculation of the affinity: 0.81×10^{-9}M (Bodman-Smith *et al.*, 2002). We decided to assess the binding of CRP to the FcγRIIA-RR allele, to FcγRIIB and to FcγRIIIB–NA2 by similar techniques. It was suspected from previous work when CRP was immobilised on plastic that such a direct immobilisation would result in potential changes in CRP binding properties (Shields *et al.*, 1991) therefore several approaches were

attempted: CRP was immobilised by ρ–aminophenyl-PCh or receptors were biotinylated and immobilised by binding to a SA sensorchip.

For the first approach, ρ–aminophenyl-PCh was immobilised through its amino group to a CM5 sensorchip. CRP was then immobilised non-covalently through binding to the PCh and finally various concentrations of FcγRs were tested for binding. No binding was seen with this approach (Figure 3.39). Different concentrations of both pCRP and rCRP were tested but no binding to FcγRIIA, IIB or RIII was observed using the conditions described.

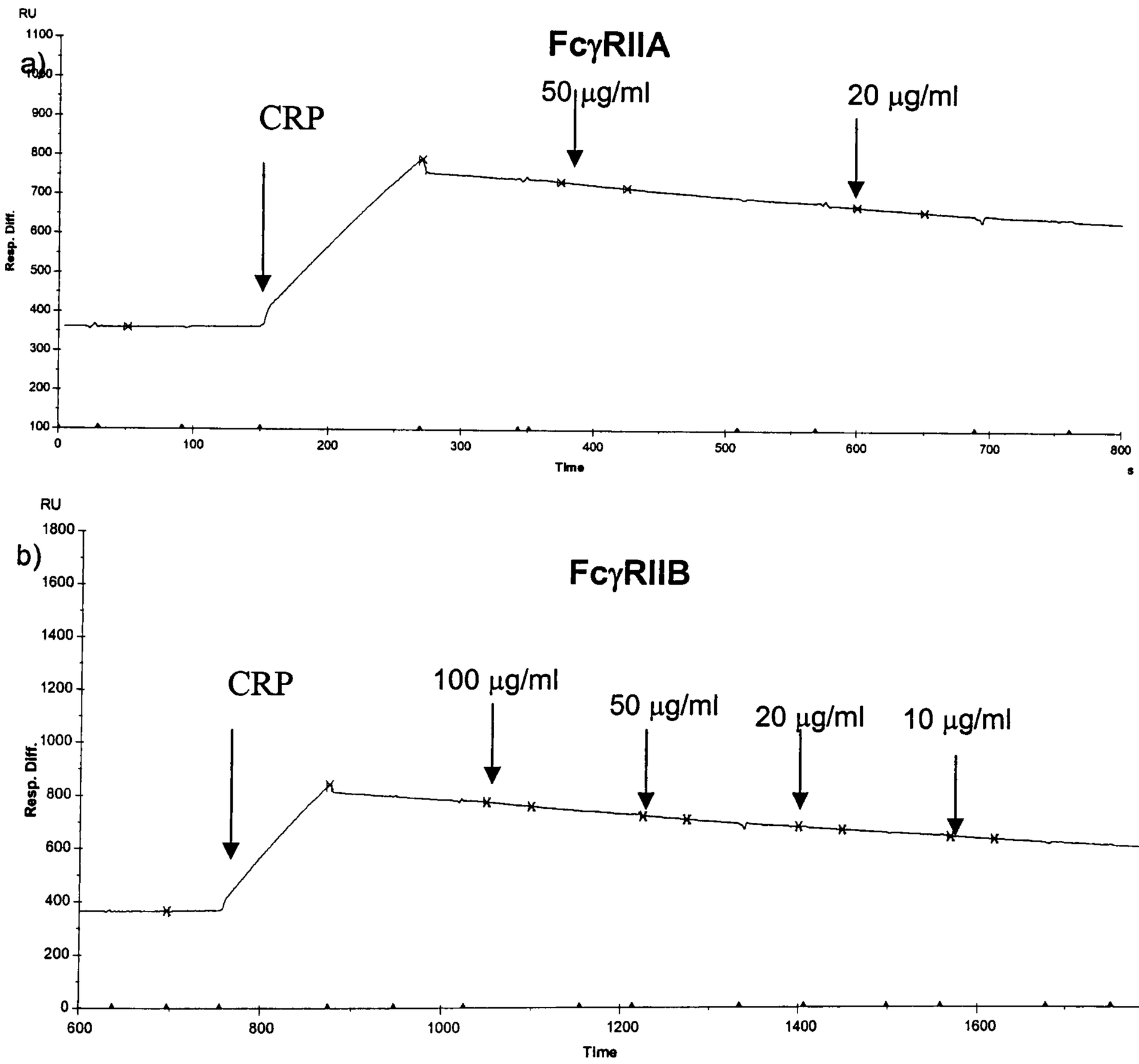


Figure 3.39. No binding of FcγRIIA or FcγRIIB to CRP bound to ρ–aminophenyl PCh. ρ–aminophenyl-PCh was immobilised on a CM5 sensorchip, then 0.5 µg/ml of CRP was added in order to yield about 500 RU bound CRP. Then a) FcγRIIA at 50 and 20 µg/ml was added and b) FcγRIIB at 100, 50, 20 and 10 µg/ml was added. Trace shows flow cell 2-1. Flow cell 1 was used as control as was described in Methods section 2.8.

One of the possibilities that accounted for the lack of binding using the above strategy is that the binding site of the receptor for the CRP may be close to the PCh binding site. Therefore a different approach was followed, by first binding FcγRs and then testing CRP binding to them. SAP, which was previously suggested to bind to mFcγRII and mFcγRIII, was also used. FcγRs were immobilised by first biotinylation using biotin N-hydroxy-succinimide and binding to streptavidin chip (SA sensorchip). Similar amounts of each receptor were bound; 3281 RU of FcγRIIB; 2805 RU of FcγRIIA and 3190 RU of FcγRIIIB.

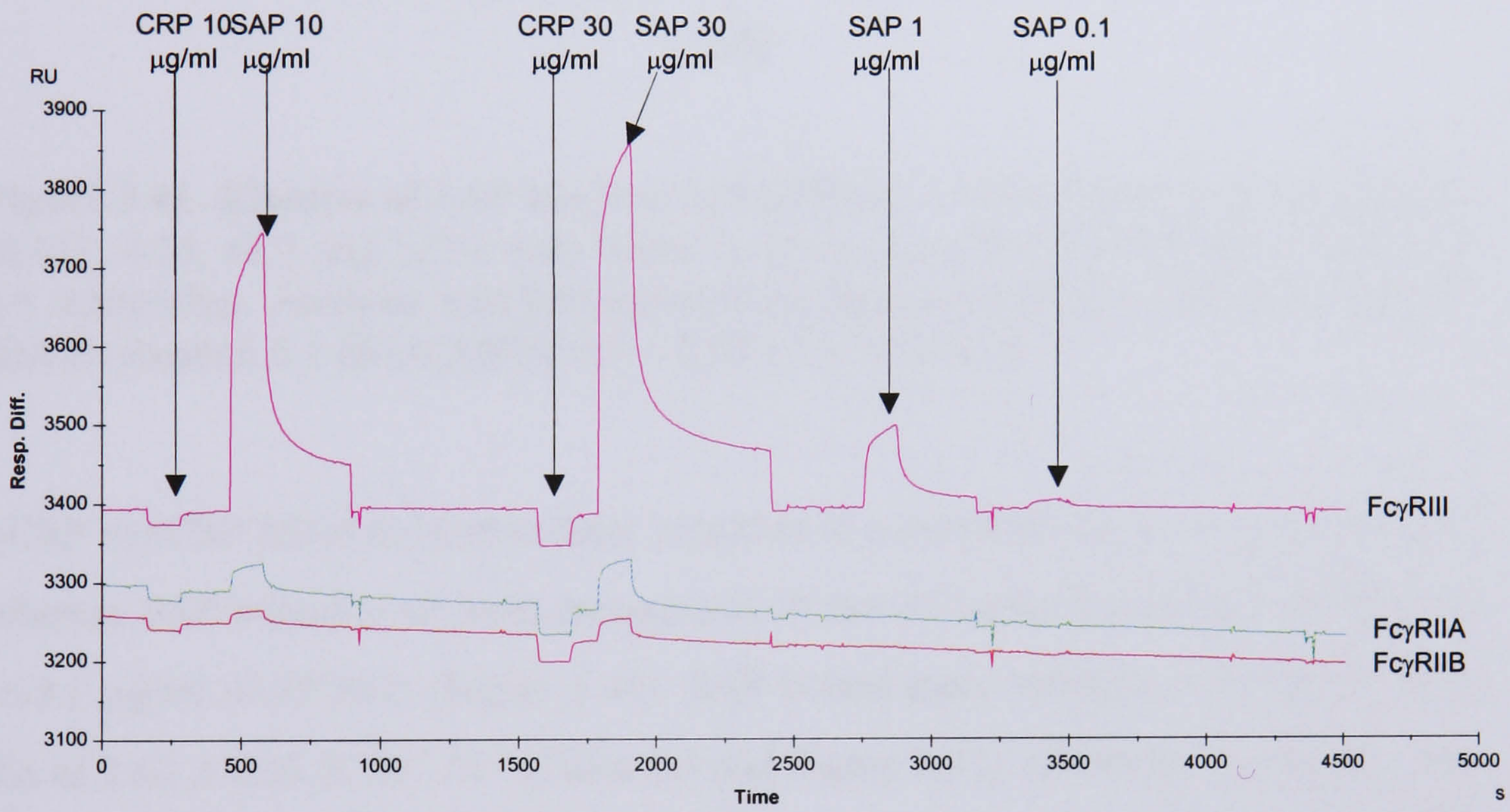


Figure 3.40. SAP binds strongly to FcγRIII. Biotinylated FcγRIIA, IIB and III were immobilised on a streptavidin sensorchip as described in section 2.8.1. CRP at two different concentrations 10 and 30 µg/ml and SAP at 0.1, 1, 10 and 30 µg/ml were injected at a flow rate of 10µl/min.

Table 3.3. SAP bound to FcγRIII. Different concentrations of SAP were analysed and the response obtained is shown (RU= response units).

SAP (µg/ml)	nM	RU
0.1	0.425	2.8
1	4.25	105.1
10	42.5	351
30	127.6	468

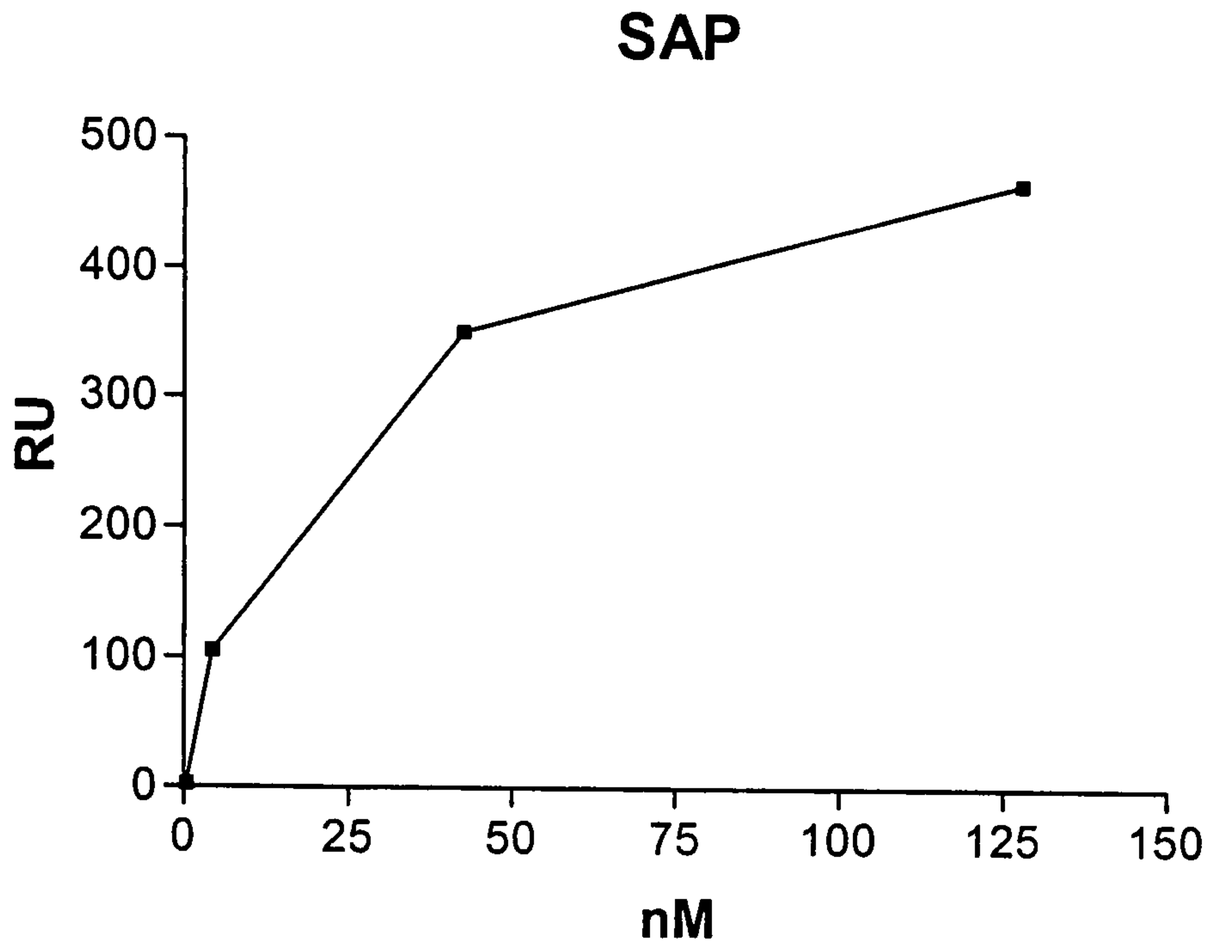


Figure 3.41. Kinetics of SAP binding to FcγRIIIB. SAP at different concentrations (0.425, 4.25, 42.5 and 127.6 nM) bound to biotinylated FcγRIIIB immobilised on a SA sensorchip. Analysis was performed using the commercially available software BIA Evaluation 2.1 (BIACORE). Kd = $2.62 \times 10^{-9} \pm 0.53\ M^{-1}$

pCRP or rCRP failed to bind to these receptors at concentrations of 10 and 20 μg/ml, whereas SAP bound to all three receptors at 10 and 30 μg/ml and bound to RIII even at 0.1 μg/ml (0.425nM) (Figure 3.40). SAP bound more strongly to FcγRIII with a Kd of $2.62 \pm 0.53 \times 10^{-9}\ M^{-1}$ (Table 3.3 and Figure 3.41). Given the potentially low affinity of CRP binding, higher concentrations might be tested. IgG binding was not tested in this system.

Although a low molar ratio of biotinylation was used, failure to observe CRP binding could be due to biotinylation of the regions of the Fc receptor required for CRP binding or to conformational alterations.

The previous experiment was only possible when the biotinylated Fc receptor had been immobilised and remaining streptavidin was bound to biotin which was passed over the column. CRP bound on its own to the SA chip, an observation that has not been reported before. CRP binding to SA was calcium dependent as addition of EDTA inhibited binding to the chip (Figure 3.42 panel a). This property of CRP was used to test binding to FcγRIIB or RIIA. However, no binding to CRP was observed at 1, 5 or 10 μg/ml of this receptor (Figure 3.42 panel b). The concentrations of CRP

were increased but none of them showed convincing binding to FcγRIIA since no significant change in slope was observed (Figure 3.43).

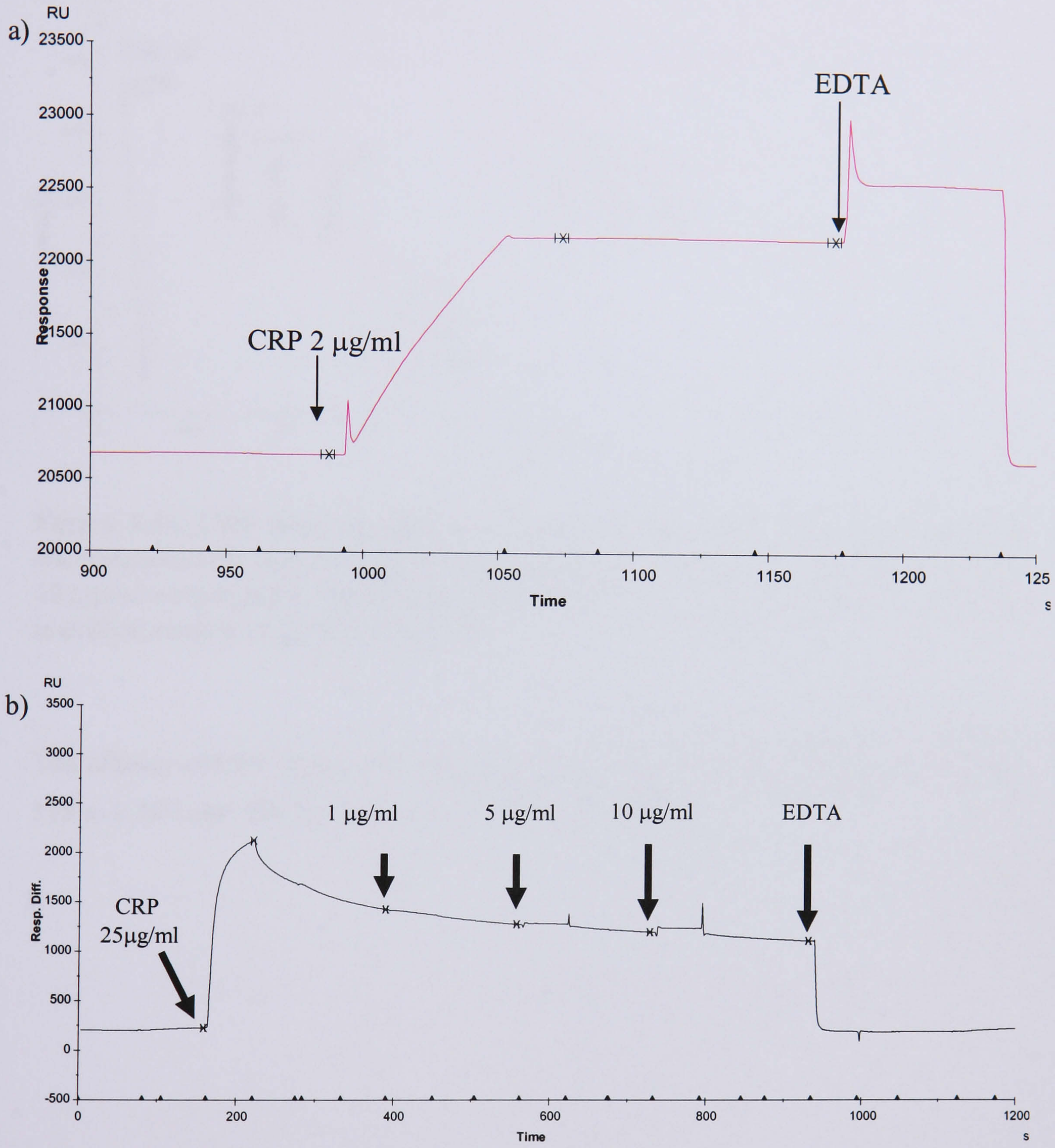


Figure 3.42. CRP binds in a calcium dependent way to the streptavidin chip. a)10 µl of 2 µg/ml of CRP at a flow rate of 10 µl/min were injected to a SA chip and binding was observed. Binding was reversed when EDTA was injected. b) Different concentrations of FcγRIIB (1, 5 and 10 µg/ml) were sequentially injected but no further binding was observed.

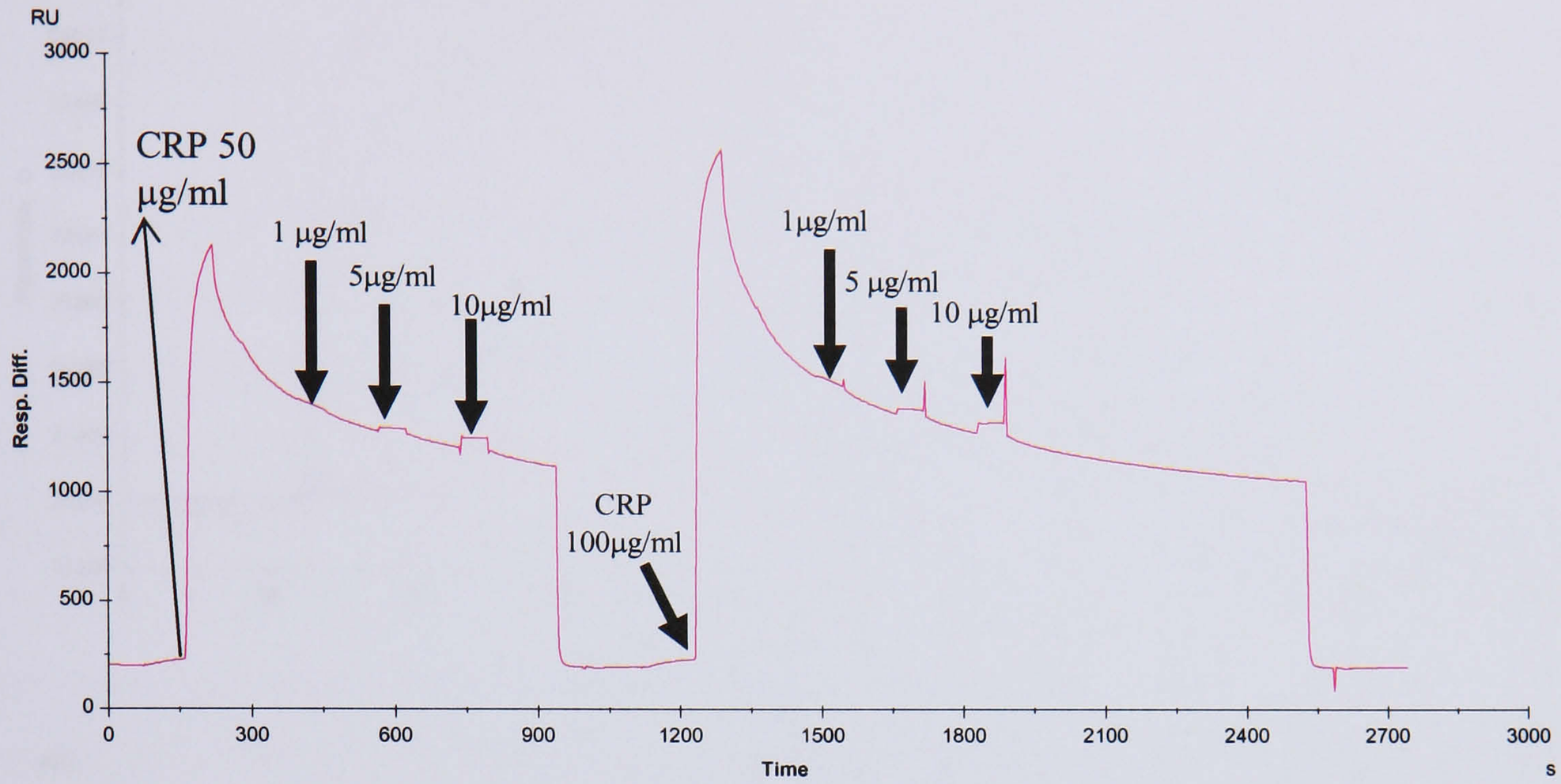
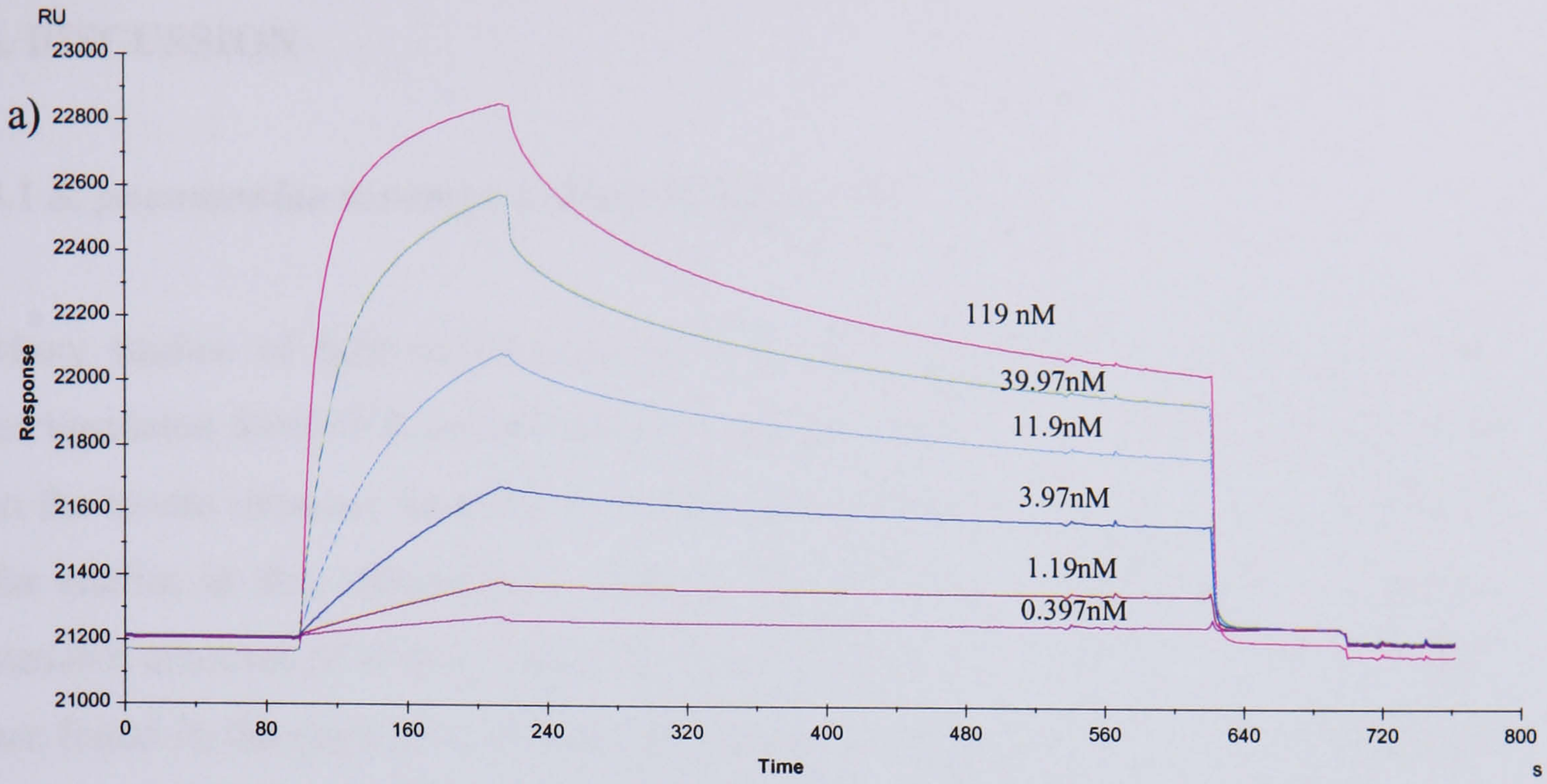


Figure 3.43. CRP does not bind to recombinant FcγRIIB. CRP at two different concentrations (50 and 100 µg/ml) was loaded onto an SA chip. FcγRIIB at 1, 5 and 10 µg/ml were added to the immobilised CRP in the presence of calcium. No binding is evident even at 10 µg/ml of FcγRIIB.

The affinity of CRP to SA was analysed using different concentrations of CRP from 119 to 0.397 nM. The K_D was 6.25×10^{-12} M (Figure 3.44).



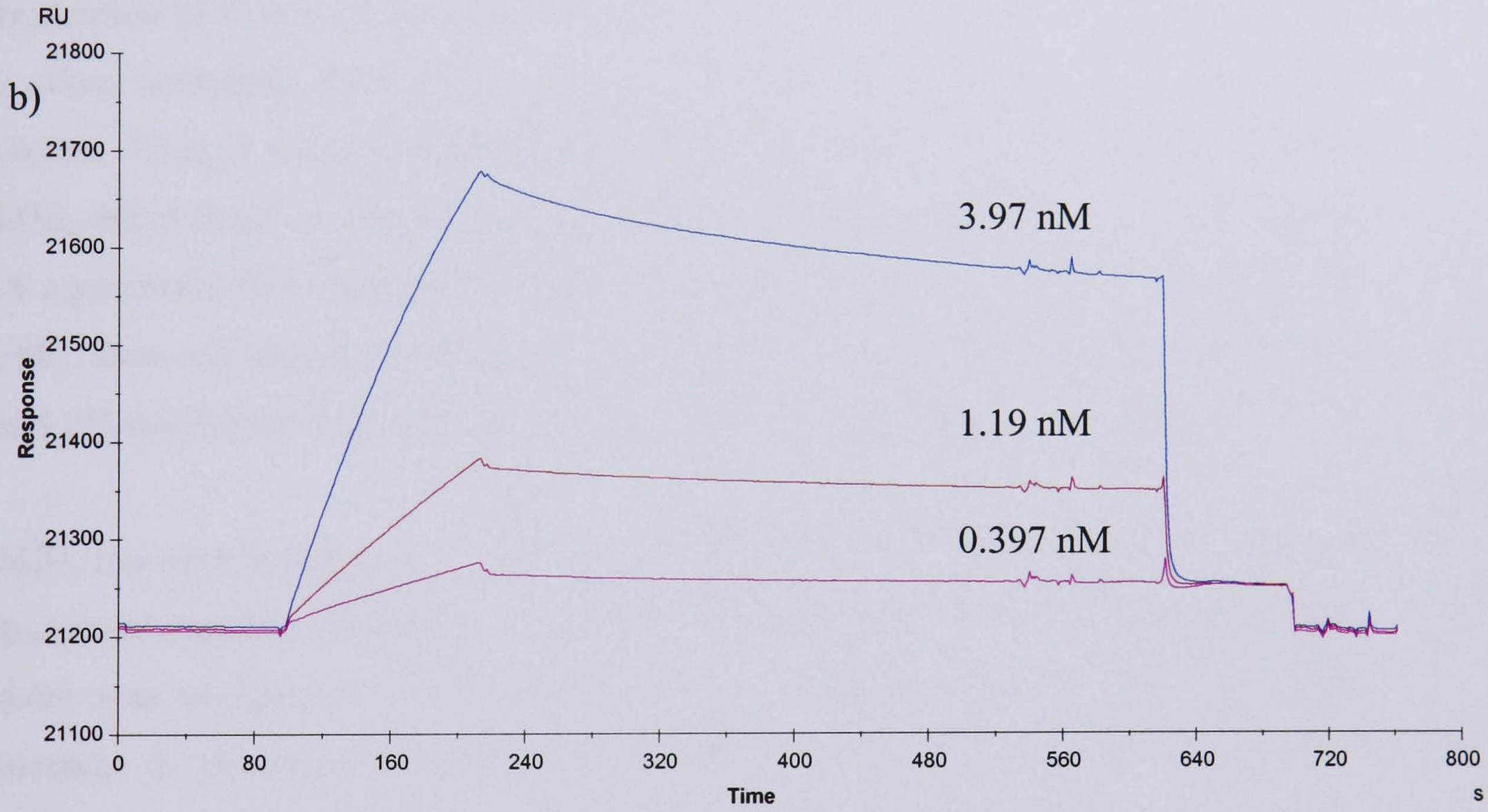


Figure 3.44 Association and dissociation curves of CRP binding to Streptavidin. Different concentrations of CRP (119, 39,97, 11.9, 3.97, 1.19 and 0.397 nM) were injected on to a SA chip at a flow rate of 30 µl/min. The Kd obtained by evaluating the 3 curves, shown in b, is about 3.25×10^{-12} M^{-1}.

4. DISCUSSION

4.1 *S. pneumoniae* serotype 3 does not bind MBL

Many studies of host interactions have used the strain R36A which is the non-encapsulated form of *S. pneumoniae* serotype 2. However, we have been interested in the innate immune response to encapsulated organisms and have used serotype 3 for studies in this laboratory. Due to the fact that the pneumococcus expresses variable amounts of N-acetyl-glucosamine in the cell wall and that a variety of sugars are found in the capsule it is likely that MBL, which binds to N-acetyl-glucosamine and mannose among other sugars, might bind to the pneumococcus and alter the production of IL-8 by neutrophils. There was no MBL binding to *S. pneumoniae* type 3 when compared with *Cryptococcus neoformans* which was used as a positive control. Thus, it was not surprising that when neutrophils were cultured *in vitro* with MBL either alone or with the pneumococcus it did not alter responses. This result is in agreement with Olaf Neth *et al.*, who showed by flow cytometry analysis that MBL does not bind to 10 different strains of pneumococcus (1, 3-6, 9V, 14,19F, 18 and 23) despite the high presence of sugars in its capsule (Neth *et al.*, 2000).

MBL has been found to bind and induce phagocytosis of *Salmonella montevideo* by human neutrophils (Kuhlman *et al.*, 1989); this effect was markedly increased when there was co-ligation with IgG (Ghiran *et al.*, 2000). Specific IgG antibodies promote *S. pneumoniae* phagocytosis and activation of neutrophils. Since IgG antibodies to *S. pneumoniae* were not used in our study, it is possible that activation of neutrophils was not fully achieved. Moreover, MBL activates the lectin pathway of complement. Thus, it is also possible that concomitant activity of MBL with C3b may induce a higher amount of IL-8 production by neutrophils; however, we did not use fresh serum to search for the role of complement on the action of MBL. The possibility remains that MBL acts on some strains of *S. pneumoniae* but not serotype 3.

Interestingly, MBL has been shown to down-regulate the expression of CD11b on neutrophils induced in response to *Neisseria meningitidis* (Jack *et al.*, 2001). The production of cytokines such as TNFα, IL-6 and IL-1β by monocytes was analysed;

MBL at 4 μg/ml increased synthesis of IL-1β and TNFα whereas production of IL-6 was decreased. MBL at 6 μg/ml or more reduced production of the three cytokines. It was interesting that different concentrations of MBL have distinct effects on the production of cytokines, with a tendency to suppress them at higher concentrations. This shows a similar effect to that seen in our finding that increasing CRP concentrations are able to down-regulate certain neutrophil functions. However, in terms of the only cytokine examined here (IL-8 synthesis) CRP upregulated the synthesis of this chemokine even at high concentrations. Such IL-8 synthesis might have a suppressing outcome on the cells (see section 4.3). The mechanism by which MBL affects the synthesis of IL-6 on monocytes is not clear, and the addition of antibody (R139) anti-C1q-MBL receptor did not convincingly affect IL-6 synthesis (Jack *et al.*, 2001). It is possible that MBL at higher concentrations recruits a down-regulatory receptor that triggers its effect on IL-1β, TNFα and IL-6.

In addition, eleven selected serum samples from children suffering recurrent respiratory infections were studied but no low concentration of MBL was detected. This observation also fails to provide a link between MBL and *S. pneumoniae* infection. Serum from children studied here, were HIV negative, and had normal IgG, IgM, IgA and IgG subclass values. Moreover, C3, C4 were also normal and the ability to produce antibodies against the pneumococcus was also normal (figure 3.5 and tables 2.1 and 3.1). Previous studies ruled out any other major humoral primary immunodeficiency in these children. So up to now the aetiology of recurrent infections in this group of children is still unknown.

In summary, our results suggest no association between MBL deficiency in the Colombian children and their observed increased frequency of pneumococcal infections, which is in agreement with a study showing that variant MBL alleles are not associated with susceptibility to invasive pneumococcal infection in randomly selected adult Danish patients (Kronborg *et al.*, 2002a) A study comparing the frequencies of alleles B, C and D of MBL and a functional promoter polymorphism at -221 (X/Y variant) with invasive pneumococcal diseases in adults reported that those homozygous for mutations of MBL who had low MBL serum concentrations may be at increased risk of acquiring pneumococcal infections (Odds ratio 2.6) (Roy *et al.*, 2002). However, a definitive study that considers analysis of concomitant

primary immunodeficiencies and epidemiological data on pneumococcal infections in children between 6 to 18 months of age, who seem to be the more susceptible to invasive pneumococcal infections, is still needed (Kronborg and Garred, 2002b).

Children of 6 – 18 months of age do not have the protective placentally transferred antibodies and their immunity has not fully matured to respond to the pneumococcal capsular polysaccharides. It is possible that a concomitant immunodeficiency in addition to MBL deficiency is required before pneumococcal infections become evident as was suggested for the first time by Turner *et al.*, (1991) and later confirmed in a group of MBL deficient children studied for recurrent infections who had higher prevalence of concomitant IgG subclass deficiency when compared with children without MBL deficiency (56% and 22% respectively) (Aittoniemi *et al.*, 1998). Later, when a group of adults with IgA deficiency was study for concomitant MBL deficiency, the frequency was not higher than the control group, probably as a result of the selection of the samples, since they were from healthy donors (Aittoniemi *et al.*, 1999). It is more likely that concomitant MBL and another immunodeficiency will be found in patients with recurrent infections.

4.2 CRP and production of IL-8 and TNFα by neutrophils

Since CRP is synthesised during the early stages (days 1-2) following an inflammatory response, its ability to modulate neutrophil function could be crucial in the initiation of immune events. Although neutrophils were once thought of purely as phagocytic cells, it has been shown that they are very active in the immune response.

IL-8 is one of the pro-inflammatory cytokines reported to be synthesised by activated neutrophils. Hachicha *et al.* (1998) showed that different organisms induce neutrophil production of IL-8. Zymosan, *Staphylococcus aureus, Salmonella typhimurium,* and *Pseudomonas aeruginosa* were the more potent inducers whereas *S. pneumoniae*, *Staphylococcus epidermidis* and *Candida albicans* were less potent. These micro-organisms were cultured with neutrophils either alone or in the presence of external TNFα, which is also able to increase the amount of IL-8 released. The authors claimed that monocytes were not a major source of IL-8 because their

preparations had less than 2 x 10^4 monocytes/ml cells and detectable levels of IL-8 were found only when monocytes were at least 5 x 10^4 (Hachicha *et al.*, 1998). Our data suggests that the capsule may be important for responses to *S. pneumoniae* since serotype 3 was able to induce IL-8 but R36A induced very little.

The ability of CRP to modify IL-8 production of neutrophils was tested when they were co-cultured with either encapsulated type 3 or non-encapsulated type 2 strain R36A *S. pneumoniae*. Unexpectedly, CRP alone was able to induce production of IL-8 by neutrophils, which to the best of our knowledge is novel. It should be remembered that for these studies we used no serum and thus CRP would have had the opportunity to interact with high affinity receptors such as FcγRI (Bodman-Smith *et al.*, 2002) or possibly with low affinity receptors such as FcγRIIA that would otherwise be blocked by monomeric IgG. IL-8 was synthesised by neutrophils and not monocytes/macrophages because of the evidence provided by intracellular FACs analysis and the absence of TNFα detected in supernatants (see below), thereby ruling out the possibility that the major source of IL-8 were monocytes and not neutrophils. This observation is interesting from the aspect of mechanisms of action but is unlikely to occur *in vivo* since IgG would inhibit such responses by competition although IgG inhibition might not be expected if CRP were bound and complexed on the surface of a micro-organism.

Neutrophils are able to promote their own chemotaxis, but this event is limited by the number of neutrophils, as shown when at higher cell density the synthesis of IL-8 is down-regulated through production of IL-1RA and soluble TNF receptors (Hattar, *et al.*, 2001). Thus, the ability of CRP to induce IL-8 by neutrophils may help them to reach a certain number of cells at the beginning of an inflammatory process up to a certain threshold. After this point, CRP at higher concentrations (>50μg/ml) may induce the synthesis of IL-1RA or other down-regulatory factors as has been reported for human mononuclear cells (Tilg *et al.*, 1993). Searching for induction of modulatory cytokines such as TGFβ by neutrophils may be of interest since this mechanism might be used by CRP to modulate the adaptive immune response.

Because CRP had been shown to bind better to the RR than the HH form of the FcγRIIA (Stein *et al.*, 2000b), we were expecting higher responses from RR than HH individuals, but that was not the case, which suggests that this polymorphism of FcγRIIA is not crucial for the modulation of IL-8 production by neutrophils. IL-8 produced in an autocrine way may affect neutrophil transmigration to extravascular sites due to changes in the cytoskeleton that decrease neutrophil deformability and therefore affects their trafficking (Harper and Savage, 2001). In this way, increased production of IL-8 induced by CRP might affect neutrophil movement to the tissues. In fact, it has been reported that CRP prevented movement of neutrophils into the lung (Heuertz *et al.*, 1993 and 1994). IL-8 synthesis by neutrophils maintained in whole blood from HH and RR donors in the presence of CRP at increasing concentrations and in the presence of different micro-organisms with different abilities to bind CRP might help to delineate the role that the synthesis of IL-8 has *in vivo*.

The ability of neutrophils to produce TNFα in response to PMA (5μM), FMLP (10μM) and LPS (up to 1 μg/ml) was analysed by ELISA. In this study the production of TNFα was consistently low, which is in accordance with Altstaedt *et al.* (1996), who showed that the main cytokine produced by neutrophils is IL-8. However, other studies have found detectable levels of TNFα: one of them, used a higher number of neutrophils per well ($5x10^5$) and also more LPS (5 μg/ml) (Dubravec *et al.*, 1990) than we used ($2x10^5$ and up to 1 μg/ml LPS). A different study used $1x10^6$ human neutrophils /well in a 24 well plate and added 1μg/ml LPS obtaining TNFα production up to approximately 250 pg/ml (Oliveira *et al.*, 1999).

This suggests that by using a higher number of cells and higher concentrations of stimuli TNFα might be detectable at low concentrations from neutrophils *in vitro*. Nevertheless, by measuring intracellular TNFα by FACs using a fluorescent antibody anti-TNFα and brefeldin A, which inhibits the secretion of the cytokine, we were unable to detect TNFα production by neutrophils. It might be that *in vivo* under different conditions and in the presence of other cells neutrophils may synthesise TNFα. We did not carry out any whole blood assays to evaluate TNFα production. It is also possible that pre-incubation of neutrophils with IFNγ alone or in conjunction

with GM-CSF may induce more production of TNFα as might occur when stimulation occurs *in vivo*. It is feasible that contamination of neutrophils with other granulocytes or mononuclear cells may have contributed to the production of TNFα observed in some reports.

4.3 CRP alters phagocytosis and NADPH oxidase activity of neutrophils

FcγRI was previously shown in our lab to be a high affinity receptor for CRP and promoted phagocytosis of CRP-opsonised SRBC (Bodman-Smith *et al.*, 2002), whereas FcγRIIA was able to bind to CRP-opsonised SRBC but phagocytosis was not markedly increased (Bodman-Smith K., Gregory R., Rodriguez J. A. and Raynes J. G. Unpublished observation). This might be due to the lack of expression of signalling molecules by Cos cells which are required for FcγRIIA mediated phagocytosis. Nevertheless, it has been seen before that FcγRIIA does not efficiently cause phagocytosis (Hutchinson *et al.*, 1995). In addition, the fact that FcγRI is present in low amounts on neutrophils might have suggested that FcγRI and FcγRIIA might combine to deliver a signal as they have been shown to do in macrophages and in transfected cells (Cameron *et al.*, 2001; Bodman – Smith K. B., Gregory R., Rodriguez J. A. and Raynes J. G. Unpublished observation).

We searched for phagocytosis of CRP-opsonised *S. pneumoniae* type 2 strain R36A and type 3 by Cos cells transfected with different FcγR combinations, but it was not possible to see clear differences between the transfected cells and non-transfected Cos cells. R36A demonstrated high background binding to Cos cells. CRP increased type 3 interactions with transfected cells but this effect was small and it was not possible to differentiate binding from internalisation in the FACS analysis despite a number of different washing steps. A reason for this failure could be that the size of the bacteria interferes with phagocytosis by these cells, which are not professional phagocytes, whereas bigger particles such as SRBC are easier to phagocytose. Carbohydrates present on the capsule or the cell wall of the bacteria which are known to decrease phagocytosis might affect non-professional phagocytic cells. *Leishmania donovani* has been shown in our lab to bind CRP (Culley *et al.*, 2000), which facilitates macrophage infection and therefore an alternative could be to infect

FcγR-transfected Cos cells to analyse to what extent the presence of a particular Fc receptor affects phagocytosis of CRP-opsonised leishmania.

Nevertheless it is important to determine whether CRP-opsonised pneumococci can be phagocytosed more efficiently by the FcγRIIA RR allele than by the HH allele because the problem could lie with specific anti-phagocytic mechanisms of *S. pneumoniae* serotype 3 whose very thick polysaccharide capsule is difficult to opsonise.

It is also possible that the presence of the PAF receptor on fibroblasts facilitates pneumococci binding to the cells and therefore it makes it difficult to differentiate between opsonised and non-opsonised bacteria. The use of pre-incubation of cells with a PAF receptor inhibitor might help to clarify if this is the case (Gould and Weiser, 2002). Finally, the use of green fluorescent protein (GFP)-expressing pneumococci might be more suitable for CRP opsonisation considering that formalin fixation may alter the expression of carbohydrates important for CRP binding to the bacteria. Unfortunately, GFP-expressed bacteria, although available, were too weakly fluorescent. It may be possible to replace the green fluorescent protein with enhanced red fluorescent protein which is a tetramer less likely to be susceptible to endogenous quenching.

The next aspect analysed was whether CRP at different concentrations had any effect on the NADPH oxidase activity of neutrophils and whether there was any difference when neutrophils from HH-homozygous individuals were compared to neutrophils from RR donors. Interestingly, increasing concentrations of CRP in the presence of *S. pneumoniae* appear to induce higher amounts of extracellular H_2O_2 and superoxide synthesis. In contrast, whilst low concentrations (approximaely 10 ug/ml) increased responses CRP at higher concentrations (e.g. 100ug/ml) down-regulated the NADPH oxidase activity as determined by DHR reduction which measures intracellular H_2O_2.

Possible explanations for this apparent contradictory effect on NADPH oxidase activity might be due to the fact that this enzyme, as well as myeloperoxidase, assembles in the plasma membrane and in the phagosome membrane, which

therefore can be regulated differently, since the phagosome membrane contains a different range of regulatory proteins and is exposed to different concentrations of signalling messengers such as calcium. The factors controlling release of oxidative radicals may be regulated differently. Such a differential regulation has been reported previously: zymosan, phorbol ester and fluoride induced the formation and accumulation of oxygen radicals intra- and extracellularly whilst ionomycin and LPS led to an intracellular accumulation in rat liver macrophages (Dieter *et al.*, 1995). On the other hand, fMLP induces mainly extracellularly released oxygen metabolites (Dahlgren 1987). Pathways for the synthesis of intracellular oxidative compounds may be different for extracellular radicals, since it was shown that PMA induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways, one dependent and one independent of PI-3K. (Karlsson *et al.*, 2003)

Our results on DHR reduction by neutrophils are in agreement with those from Buchta *et al.*, (1987) who found that CRP at concentrations between 10 and 100 μg/ml was able to down-regulate superoxide production induced by PMA, when CRP and PMA were added together, although this group used extracellular determination of the activity. However, at higher concentrations CRP may be suppressing neutrophil activity as shown by Müller and Fehr (1986) who treated neutrophils with increasing amounts of CRP up to 300 μg/ml and observed a decreased stimulation of the hexose monophosphate pathway activity by generation of $^{14}CO_2$ from [^{14}C] glucose. Furthermore, Tatsumi *et al* (1988) showed that at 100μg/ml CRP was able to suppress neutrophil chemiluminescence by luminometry induced by PAF. This technique allows one to search for superoxide production at both the intra and extracellular level (Dahlgren and Karlsson, 1999) (Table 4.1).

CRP at high concentration inhibited the intracellular reactive oxygen of primed neutrophils and also inhibited responses to PMA but not to fMLP or LPS. This selectivity may relate to the specific pathways activated by PMA which as mentioned before may be different from the ones used by other stimulants. Intracellular reactive oxygen in response to PMA has been reported to be dependent on PI-3K activity whereas extracellular production of superoxide was independent (Karlsson *et al.*, 2000). It is possible that CRP at acute-phase concentrations may lead to activation

of SHIP which would reduce the effects of PI-3K activation. It is known that SHIP is recruited by FcγRIIB ligation. Two other activators of intracellular but not released reactive oxygen radicals such as ionomycin (Dahlgren 1987) and CR3 ligation (Serrander *et al.*, 1996) might be examined to test whether CRP is able to modulate their activity.

Moreover, others have found that when fMLP was used to induce p38 MAPK and chemotaxis, this response was inhibited by CRP at concentrations higher than 10 μg/ml (Heuertz *et al.*, 1999). When the published literature is reviewed for the effects of different concentrations of CRP on neutrophil responses, it is evident that CRP at lower concentrations has effects which are quite distinct from those induced by higher concentrations (Table 4.1).

Table 4.1 Different neutrophil responses to various CRP concentrations. CRP at increasing concentrations tends to down-regulate the function of neutrophils analysed, whereas lower concentrations tend to give the opposite effect.

Function analysed	CRP (μg/ml)	Response obtained	Reference
- Hexose monophosphate shunt activity by quantifying $^{14}CO_2$ release - Chemotaxis	100 –300	↓ at increasing concentrations ↓ from 200 μg/ml	(Muller and Fehr, 1986)
- O_2^- production by reduction of cytochrome c - Chemotaxis toward fMLP	0.1-100	↑< 5 ↓>5μg/ml ↑0.1 to 1μg/ml ↓> 1	(Buchta *et al.*, 1987)
Chemiluminescence induced by PAF	≤100	Suppressed	(Tatsumi *et al.*, 1988)
- Chemotaxis toward fMLP and IL-8 - P42/44 MAPK, PI3K	10, 20 and 100	↓ ≥10 ↑ ≥10	(Zhong, *et al.*, 1998)
- Chemotaxis toward fMLP - P38 MAPK	0 – 1000	↓> 10	(Heuertz, *et al.*,1999)

No reports on effects of different concentrations of human CRP on phagocytosis by neutrophils were found. In a study where phagocytosis of *S. pneumoniae* serotypes 7F and 4 opsonised with CRP at different concentrations were compared with antibodies against capsular polysaccharides, it was concluded that CRP did not increase opsonic activity of these serotypes (Chudwin *et al.*, 1985), a close examination of this reference revealed that those conditions with CRP at high concentrations had lower phagocytosis which correlated with our findings.

CRP has been claimed to exhibit a protective activity against *S. pneumoniae* in mice (Mold *et al.*, 1981), but more recently the FcγRIIA allelic form has also been linked to resistance (Shelley Segal, Oxford, personal communication). However, the gene for CRP is found close to the genes for Fc receptors on chromosome 1q (within 1.8 Mb) and other immune response genes making data difficult to interpret. It has been proposed that SHIP might be activated through FcγRIIA, which might explain down-regulatory effects upon binding to CRP (Tridandapani *et al.*, 2002b). However, this possible pathway remains to be clarified.

Both isolated neutrophils and neutrophils maintained in whole blood gave similar results in which CRP at increasing concentrations decreased the NADPH oxidase activity determined by DHR. This argues against effects being only apparent on neutrophils following any stimulatory effects on neutrophils caused during isolation procedures. One concern that we had with regard to the use of citrate plasma was the well known ability of citrate to chelate calcium. However, reference to several papers on the levels of the free calcium in citrated plasma revealed that 0.05 – 0.1 mM was typical (Bode *et al.*, 1989). This amount of calcium would be sufficient to support CRP binding to *S. pneumoniae*.

One of our concerns was that the down-regulatory effect was due to apoptosis induction by CRP, but CRP did not alter apoptosis on neutrophils. We also analysed the effect of pneumococci and found that they also induced necrosis although that effect did not seem to be altered by the presence of CRP. Therefore we are confident that the responses observed were not due to any dysfunction of the neutrophils due to

CRP induced apoptosis. As previously observed the *S. pneumoniae* themselves were able to induce increased apoptosis (Engelich *et al.*, 2001).

Modified CRP (mCRP) which can be formed from the pentameric form by treatment with 8M urea in the presence of 10mM EDTA for 12 hours at 37°C, has been reported to have different effects than the native form (Potempa *et al.*, 1988). mCRP from 1 μg/ml to 100 μg/ml, but not native CRP, upregulated the expression of CD11b/CD18 and delayed neutrophil apoptosis. These effects were due to activation of the Erk pathway since it was sensitive to the presence of PD98059 (an inhibitor of this pathway) (Zouki *et al.*, 2001; Khreiss *et al.*, 2002). Although it may be possible that under certain conditions formation of mCRP may occur *in vivo*, the real significance of mCRP remains to be seen. In any event our purified CRP did not demonstrate any similar effects.

One explanation for the down-regulatory effects for CRP on neutrophils reported under different conditions could be FcγRIIB ligation. It is reported that mRNA for FcγRIIB is found in human neutrophils (Cassel *et al.*, 1993) and that in mice it is thought that CRP binds to FcγRII (Stein *et al.*, 2000a) (mice only express FcγRIIB), we searched for the expression of FcγRIIB on neutrophils and tested the possibility that CRP is able to bind to human FcγRIIB and contribute to the effects seen. CRP is also able to decrease binding of neutrophils to endothelial cells, an effect seen at high concentrations of CRP and related to the shedding of L-selectin, with lack of upregulation of CD11b/CD18. This effect might also inhibit neutrophil movement to the tissues (Zouki *et al.*, 1997; Zouki *et al.*, 2001).

Despite some experiments which seem to suggest the expression of FcγRIIB on neutrophils we were unable to confirm the above proposal. The main reason for this is the lack of specific reagents to differentiate between FcγRIIB, RIIA and RIIC; an alternative could be to immunoprecipitate FcγRIIB from the plasma membrane of neutrophils and then search for SHIP activity which can be detected by phosphorylation of a specific substrate. It is certain, however, that reagents for FcγRs remain a problem for many aspects of Fc receptor biology. The similarity of the

external region of different receptors and failure to use $F(ab')_2$ fragments have led to uncertainty in many studies.

Macrophages are known to increase the expression of FcγRIIB in the presence of IL-4 (Tridandapani *et al.*, 2002a). Conversely, IFNγ increases FcγRI and decreases FcγRIIB (Pricop *et al.*, 2001). The possibility that CRP acts through FcγRIIB might therefore be tested by comparing the ability of CRP to down-regulate NADPH oxidase activity or phagocytosis in normal versus IL-4-treated and IFNγ-treated cells. In the same way, CRP at acute-phase concentrations might down-regulate phagocytosis of zymosan or of C3b-opsonised particles by neutrophils or macrophages, which would add to the capacity of acute-phase CRP to interfere with other receptors involved in phagocytosis. Further studies in future could attempt to incorporate constructs of FcγRIIB into primary cells to look at function, either by introducing active FcγRIIB or alternatively by introducing dominant negative or RNAi inhibitory constructs of FcγRIIB in vectors such as psectag 2 in order to deplete functional FcγRIIB.

Although B cells express a different variant of the FcγRIIB (FcγRIIB2), it is possible that CRP may alter the production of antibodies by these cells. Experiments designed to search for this possibility may provide an interesting clue for the role of CRP within the adaptive immune response. Early studies on CRP did reveal effects on antibody responses, CRP at 10 and 25 μg/ml increased production of B cell colony formation whereas CRP at 50 μg/ml decreased colony formation when compared to controls in the absence of CRP (Whisler *et al.*, 1983).

4.4 CRP and SAP binding to FcγRs as studied by surface plasmon resonance

We immobilised recombinant human FcγRIIB to analyse binding using Biacore®, however we did not see binding to CRP. One possible explanation for this result is that normally in the cell transfection experiments in mammalian cells the FcγRIIB is highly glycosylated. Absence of glycosylation on the *E. coli* expressed recombinant form may alter binding to CRP. Moreover, a different group using the same FcγRs provided by Dr P. Sondermann found that CRP immobilised on a CM5 chip did not

bind FcγRs (Dr Young, Birmingham, personal communication). This approach may alter CRP structure as was demonstrated for CRP adsorbed to plastic surfaces where antibodies against CRP treated with urea (neoantigen CRP) were found to bind plastic-absorbed CRP but not native CRP (Potempa *et al.*, 1987). However, IgG binding to FcγRIIB using a similar methodology has previously been reported (Mimura *et al.*, 2001). In the reverse direction IgG glycosylation has been shown to be required for Fc receptor recognition as it allows flexibility of the FcR-IgG complex in contrast to lack of glycosylation which renders the contact loop more rigid and therefore reduces the affinity of binding (Krapp *et al.*, 2003). Interestingly the interaction between IgA and FcαRI has recently been shown to involve carbohydrate on the Fc receptor domain (Herr *et al.*, 2003). Alternatively, the carbohydrate may merely be required for maintaining the correct orientation of domains. Using FcγRIIB expressed on liposomes in the Biacore system may provide a different tool to search for CRP binding to this receptor.

The apparent requirement for receptor glycosylation for CRP binding suggests that it may be necessary to generate a GPI-anchored protein that attaches to the surface of a cell, such as Cos cells, via a phosphatidyl inositol linkage. This glycosidic bond can be enzymatically hydrolysed using phosphatidyl inositol phospholipase C, which liberates the protein from the surface (Harrison *et al.*, 1998). A similar glycosylated protein has been immobilised on a CM5 chip using a specific antibody to search for CRP binding (Bodman-Smith *et al.*, 2002).

Although binding of SAP to human neutrophils was reported previously (Landsmann *et al.*, 1994) the nature of the receptor was unknown. Subsequently, binding of SAP to mFcγRI and mFcγRIII but not FcγRII was reported (Mold *et al.*, 2001). From our experiments using surface plasmon resonance it is clear that SAP binds to human FcγRIIIB, which is novel, although it is not known if the polymorphisms described on FcγRIIIB might affect the binding of SAP. This could be investigated using the same technique. Previous studies showed that FcγRI expressed by Cos-cells transfected with a GPI-FcγRI construct and thus in a glycosylated form did not bind human SAP. This is at variance with the studies of Mold *et al.*, (2001).

In addition our data did suggest that the FcγRIIA and FcγRIIB did interact weakly with SAP. The binding to FcγIIIB was calcium dependent as shown previously for binding of CRP to FcγRI (Bodman-Smith *et al.*, 2002). The relevance of this is unclear but may indicate that binding to receptor is at or close to the calcium dependent binding site, but it is also known that loss of calcium causes considerable alteration to neighbouring loops in the CRP subunit (Shrive *et al.*, 1996). The biotinylation of receptor and immobilisation apparently leaves only a small proportion of the receptor immobilised in an orientation or conformation capable of binding SAP. Therefore we are unable to draw any conclusions about where SAP binds as a pentamer or decamer.

Interestingly, and somewhat surprisingly, CRP bound to the SA chip, a finding that has not been reported before. A possible explanation for this is the presence of an amino acid motif in the CRP similar to the biotin-like motif HPQ (Figure 4.1), which has been reported to insert itself into the biotin-binding cleft forming hydrogen bonds and hydrophobic interactions with SA (Schmidt *et al.*, 1996). Different peptides containing this sequence were found to bind SA (Katz 1995; Wilson *et al.*, 2001).

The observation that biotin was able to inhibit CRP binding to the SA chip and that SAP (which does not contain two amino acids of this crucial motif) was unable to bind to SA (Figure 4.1) suggests that the presence of this motif is important in explaining SA binding. Experiments to confirm this will involve competition with synthetic peptides. Examination of the structure (Shrive *et al.*, 1996) revealed that this peptide is close to the intersubunit interface, but the sequence is on a surface that in CRP extends away from the surface compared with SAP. The possibility that interactions occur with other proteins that bind biotin in mammalian or bacterial systems is being investigated. An additional use of this finding might be in the purification of CRP from plasma or in serum CRP assays for clinical diagnostics.

Peptide "biotin-like"	-		F	S	H	P	Q	N	T	
CRP		-E	V	F	T	K	P	Q	L	W	P
SAP		-Y	V	I	I	K	P	L	V	W	V

Figure 4.1 Presence of an HPQ-like amino acid motif in CRP. Alignment of sequences found in biotin, CRP and SAP. CRP contains a KPQ motif similar to the

HPQ motif present in peptides able to bind SA. SAP has a sequence that differs from the one found in biotin and CRP.

A recent paper has suggested that contaminant proteins and in particular IgG in preparations of CRP may account for some of the functions ascribed to it (Hundt *et al.*, 2001). This was always unlikely since the antibodies present would require the ability to bind to PCh in a calcium dependent manner. To deal with this potential problem, we analysed neutrophil responses in the presence of both rCRP and pCRP which gave essentially similar results. We tested our pCRP on an SDS-PAGE to see if further bands were observed, but only the one corresponding to CRP was seen (Figure 3.22) and no IgG was observed by immunoblotting. rCRP has been reported to be contaminated with low levels of LPS but we consider this unlikely to cause the responses seen since no DHR response was observed in neutrophils with 10 ng/ml LPS, an amount in excess of that which was present in our pCRP (Figure 3.25).

Since increased CRP serum concentrations and numbers of neutrophils arise simultaneously at sites of tissue inflammation, it is reasonable to try to identify the possible effect that CRP has on the function of these cells. However, there are controversial reports from the groups that have been working in this field. There are many factors that intervene in the interaction of CRP with neutrophils that may help explain the variable results obtained:

1. CRP has more than one receptor on neutrophils. It has a strong interaction with FcγRI, and may also bind to the FcγRIIA R and FcγRIIB alleles. It is possible that FcγRI may signal through FcγRIIA rather than the γ chain since FcγRI and FcγRIIA co-transfected-Cos cells are able to phagocytose both IgG and CRP-opsonised SRBC but transfection of FcγRIIA alone does not allow phagocytosis (Bodman-Smith *et al.*, personal communication). It is also possible that a different receptor exists besides these two receptors. Evidence for this has been reported recently since CRP alone showed binding to FcγRIIA on macrophages although this was determined using a complete FITC-labelled antibody anti-CD32, which would also bind to FcγRs through its Fc portion. CRP complexed with low density lipoprotein binds to an as yet unidentified receptor (Fu and Borensztajn, 2002). Many other negative signalling receptor complexes such as

CD45, CD47 or PTP recruit SHIP and the possibility cannot be ruled out that CRP interacts with one of them (Figure 4.2).

2. The binding of the receptors to CRP may differ according to genetic polymorphisms and this property may explain differences found by different authors. Studies here however suggest that the H131R polymorphism of FcγRIIA is less important that previously suggested.
3. The state of activation of neutrophils is also important since neutrophils are very sensitive to different procedures of isolation and activation. After activation with IFNγ neutrophils increase the amount of FcγRI expressed on their surface after 18-hour incubation (Hoffmeyer *et al.*, 1997), and freshly isolated neutrophils from infected patients have been shown to express FcγRI (Guyre *et al.*, 1990). Since FcγRI is one of the possible receptors for CRP its expression and co-ligation may alter the functional outcome in the neutrophils.

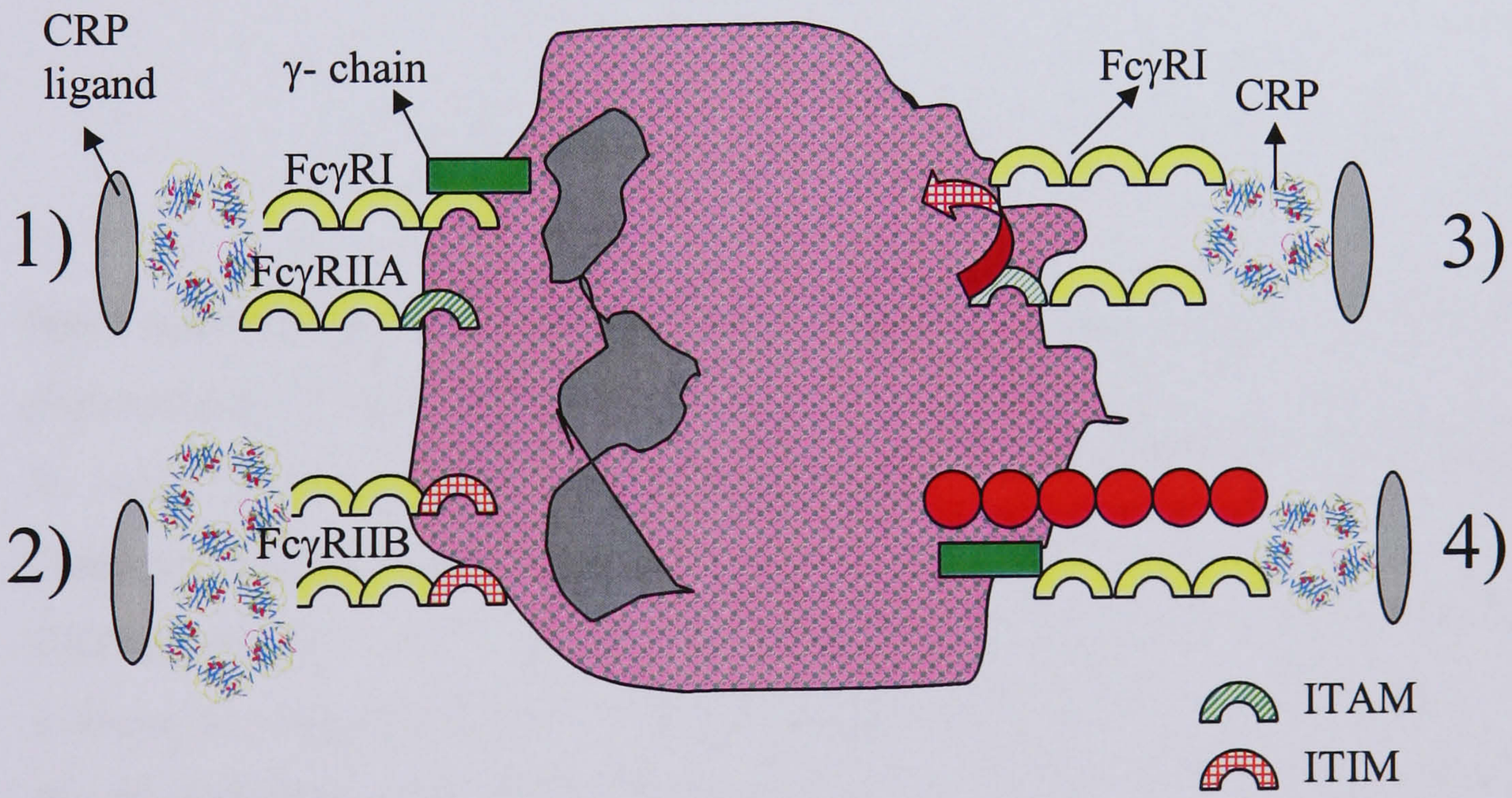


Figure 4.2 Model for human CRP binding to neutrophils. 1) CRP at low concentrations binds to either FcγRI with high affinity or to FcγRIIA with low affinity upregulating neutrophil function. 2) CRP at higher concentrations may bind to FcγRIIB down-regulating neutrophil function. 3) It is possible that a down-regulatory pathway may exist directly from FcγRIIA. 4) Alternatively CRP can bind to an as yet unidentified receptor (depicted by red circles) that can trigger a down-regulatory response.

4. Neutrophils also express an FcγRIIC molecule which contains two extracellular domains from RIIB and intracellular domains from RIIA (Warmerdam *et al.*, 1993). mRNA for FcγRIIC has been shown in human neutrophils (Cassel *et al.*, 1993) and some isoforms of this molecule have been reported in NK cells which may or may not have an ITAM molecule in their intracellular domain (Metes *et al.*, 1999). If FcγRIIC is able to bind CRP, which might be predicted because of the similarity to FcγRIIB, its expression on neutrophils may alter the response observed between different donors.

5. The observed effects on neutrophil activity might relate to the presence of *S. pneumoniae* and for that reason experiments analysing neutrophil physiology in the presence of other particles or micro-organisms opsonised with different concentrations of CRP might clarify to what extent pneumococci are involved in a particular response.

CONCLUSIONS

1. MBL does not appear to play a major role in neutrophil recognition of the *S. pneumoniae* serotypes used in this study.
2. *In vitro* TNFα production by neutrophils was not detectable by the ELISA technique used or by intracellular staining.
3. CRP on its own or with *S. pneumoniae* does not increase apoptosis of neutrophils whereas *S. pneumoniae* type 3 induces both apoptosis and necrosis of these cells.
4. For the first time it was shown that human CRP was able to induce the synthesis of IL-8 on human neutrophils in serum free conditions.
5. Despite the observation that CRP binds to FcγRIIA R131 but less to H131 in transfection assays, there is no evidence that this has a role in neutrophil responses to CRP. This either questions whether FcγRIIA has an activating function in human neutrophils or whether the differences in CRP binding to allelic forms of the receptor are sufficient to give rise to differences in response.
6. CRP at low concentrations increases phagocytosis and NADPH oxidase activity

7. CRP at high concentrations has different effects on intracellular and plasma membrane located NADPH oxidase activity decreasing the former and increasing the latter.
8. CRP at high concentrations does not increase phagocytosis of *S. pneumoniae* type 3
9. The above effects are not due to IgG contamination since similar responses have been obtained with recombinant CRP
10. SAP binds to human FcγRIII with a Kd of $2.62 \pm 0.53 \times 10^{-9}$ M^{-1}.
11. CRP bound to streptavidin and this effect could be inhibited by the presence of biotin.

FUTURE WORK

1. Since CRP is able to down-regulate some neutrophil functions, future work could analyse the ability of CRP to down-regulate responses induced by different receptors such as complement receptors or IgG mediated responses through the same receptor. CRP used at increasing concentrations might also down-regulate responses induced through CR3 or CR4 on neutrophils.

2. In order to test CRP binding to FcγRIIB, it may be necessary to generate glycosylated forms of this receptor for surface plasmon resonance studies of CRP binding. However, there is no certainty that this receptor is expressed by human neutrophils.

3. It is possible that CRP at increasing concentrations is able to induce the synthesis of cytokines such as TGF-β on neutrophils. This or other regulatory cytokines might be explored in order to search for other mechanisms by which CRP is able to modulate neutrophil functions.

4. One possibility that has not been considered is the interaction of CRP with PAF. This interaction is known to occur and could have effects in this system in several ways. For example perhaps high CRP concentrations could prevent autocrine actions of PAF. *S pneumoniae* is known to interact with

PAF receptors so that other possible effects might be observed in the presence of bacteria. It would be possible to determine if PAF were involved using PAF antagonists.

5. CRP may alter the presentation of antigen to which it binds, so studies that search for CRP and antigen presentation by dendritic cells to T cells may offer a different perspective of the functionality of this versatile molecule.

6. Moderately elevated CRP is a risk factor for heart disease and CRP binds to oxidised LDL. If the pattern of uptake seen in neutrophils was the same in monocytes/macrophages then these observations suggest that increased phagocytosis at intermediate (upper limit of normal) concentrations of CRP would be potentially important in the phagocytosis of oxidised LDL into monocyte/macrophages. Therefore it would be valuable to test the ability of CRP to alter phagocytosis of oxidised LDL.

7. The interaction between streptavidin and CRP is very interesting and if CRP resembles biotin, the possibility is that it interacts physiologically not with streptavidin but another biotin binding protein, possibly a transporter. Biotin availability is known to alter proliferation of lymphocytes.

8. The ability of SAP to bind FcγRIIIB could lead to activatory or inhibitory functions on neutrophils. Initially, the effect of SAP on immune complexes which work through the receptor could be examined.

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